HDL proteomic signatures as potential biomarkers of cachexia and sarcopenia in gastrointestinal cancer.

This thesis is submitted to University College Dublin in fulfilment of the requirements for the degree of Doctor of Philosophy (PhD).

Rianna McElroy

19209434

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School of Public Health, Physiotherapy, and Sports Science

University College Dublin

Head of School: Professor Catherine Blake

Supervisor: Professor Helen Roche, **Co-supervisor**: Assistant Professor Fiona McGillicuddy

Doctoral Studies Panel: Prof. Helen Roche, Assist. Prof. Fiona McGillicuddy, Prof. Aoife Ryan, Assoc. Prof. Gerard Cagney

Statement of Original Authorship

I hereby certify that the submitted work is my own work, was completed while registered as a candidate for the degree of Doctor of Philosophy, and I have not obtained a degree elsewhere based on the research presented in this submitted work.

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Rianna McElroy , September 2024

Author Contributions and collaborations:

Chapter 1

Title: Circulating Biomarkers of Cachexia and Sarcopenia in Gastro-intestinal cancer patients: A Rapid Review

Authors: Rianna McElroy, Erin S. Sullivan, Kathleen A. J. Mitchelson, Aoife Ryan, Fiona C. McGillicuddy, Helen M. Roche

Contributions: H.M.R, F.C.McG and A.R, developed the concept, study hypothesis and design. H.M.R reviewed and edited the manuscript. R.McE aided in the study design, developed and conducted the search, screened papers, extracted data from selected papers and drafted the manuscript. E.S.S. reviewed the data extraction table and provided guidance. K.A.J.M provided support for preliminary work.

Chapter 2

Title: Modification of the protein cargo of HDL particles in patients at risk of sarcopenia – a novel sensor to guide precision nutrition interventions

Authors: Rianna McElroy, Caoileann Murphy, Rachel Byrne, Helen M Roche, Fiona C. McGillicuddy

Contributions: H.M.R and C.M developed the concept, study hypothesis and design of the original Nutrimal study from which the samples in the present study were derived. H.M.R, C.M and F.C.McG developed the concept, study hypothesis and design of the presented study. F.C.McG and H.M.R also evaluated the data, aided with the manuscript and provided overall supervision. R.McE performed all experiments and data analysis, interpreted the results and drafted the manuscript of the presented study. R.B provided training and support.

Chapter 3

Title: High Density Lipoprotein proteomic signatures as potential biomarkers of cachexia and sarcopenia in a cohort with gastrointestinal cancer

Authors: Rianna McElroy, Erin S. Sullivan, Rachel Byrne, Aoife Ryan, Fiona C. McGillicuddy, Helen M. Roche

Contributions: H.M.R, F.C.McG, E.S.S and A.R developed the concept, study hypothesis and design of the presented study. E.S.S and A.R managed the original study from which the samples were derived. R.McE performed the experiments, data analysis, interpreted the data and drafted the manuscript. R.B provided technical support, training and feedback. F.C.McG and H.M.R aided in drafting and editing the manuscript and provided overall supervision.

Chapter 4

Title: HDL Proteome Remodelling and Hepatic Alterations in a Mouse Model of Cancer Cachexia

Authors: Rianna McElroy, Morgane M. Thibaut, Laure B. Bindels, Fiona McGillicuddy, Helen M. Roche

Contributions: H.M.R, F.C.McG, M.M.T and L.B.B developed the concept, study hypothesis and design of the presented study and provided supervision. M.M.T and L.B.B managed the original study from which the samples were derived. R.McE did the experiments, data analysis and interpretation and drafted the manuscript. H.M.R edited the manuscript and provided overall supervision.

Conferences, presentations, courses and awards

Conferences:

- ESPEN-Virtual Congress On Clinical Nutrition And Metabolism **2020**
- Keystones-eSymposia Proteomics in Cell Biology and Disease **2020**
- European And International Congress On Obesity Online **2020**
- 13th International Conference on Sarcopenia, Cachexia, & Wasting Disorders -congress online **2020**
- 14th International Conference on Sarcopenia, Cachexia, & Wasting Disorders **2021**
- Institute of Food and Health Research Showcase **2022**
- Irish Association for Cancer Research-58th Annual Conference **2022**
- Irish Association for Cancer Research-59th Annual Conference **2023**
- IrSPEN- Fit to function: a new focus on muscle in aging and disease **2023**
- 16th International Conference on Sarcopenia, Cachexia, & Wasting Disorders **2023**
- 7th Cancer Cachexia Conference **2023**
- Keystones-Cachexia and wasting syndrome in cancer and chronic diseases **2024**

Presentations:

- HDL associated biomarkers of sarcopenia/dynapenia, Institute of Food and Health Research Showcase poster (2022), UCD, Dublin, Ireland **(poster)**
- HDL associated biomarkers of sarcopenia/dynapenia, Irish Association for Cancer Research-59th Annual Conference (2023), Athlone, Ireland **(poster)**
- HDL biomarkers of muscle wasting and weight loss in cancer, Diabetes complications research centre (2023), UCD, Dublin, Ireland **(oral)**
- HDL biomarkers of Cachexia and Sarcopenia in gastrointestinal cancer, Keystones-Cachexia and wasting syndrome in cancer and chronic diseases (2024), San Francisco, California, United States **(poster)**

Awards:

- Best poster award- Institute of Food and Health Research Showcase poster (2022), UCD, Dublin, Ireland.
- Travel reimbursement award-Keystones-Cachexia and wasting syndrome in cancer and chronic diseases (2024)

Courses:

- ESPEN Life Long Learning Webinar Course-Nutritional Support in Cancer **2020**
- ESPEN Life Long Learning Webinar Course-Nutritional in the Older Adults **2020**
- The Nuriton Society-Nutrition and exercise interventions in cancer and cachexia **2021**
- Introduction to ÄKTA™ chromatography systems (ÄKTA pure™ & ÄKTA avant™) and UNICORN™ 7.9 control software **2023**

General Thesis Abstract

Cancer cachexia is an inflammatory condition characterised by rapid weight loss (skeletal muscle and/or fat mass) that affects up to 80% of patients with cancer. It severely impacts patients quality of life, leading to reduced treatment tolerance and survival. Despite its prevalence, the condition is severely underdiagnosed, partially due to a lack of awareness and consensus definition, but also due to difficulties with screening, such as reliance on prior weight history and the cost and time required for muscle quantification. Therefore, a blood based biomarker that will make the screening of cachexia, easy, routine and actionable is sorely needed. However, despite the large number of candidate markers identified in the literature, none have made it to clinic. This is due to a lack of specificity, reproducibility issues, and for some, difficulties with measurement. In this thesis, we propose a novel biomarker in the form of the High Density Lipoprotein (HDL) proteome. HDL are protein rich particles and previous research has shown that they are affected in inflammatory and metabolic disorders such as cardiovascular disease and diabetes.

In study 1, we investigated the HDL proteome in a subset of the Nutrimal study which investigated the effects of Leucine (Leu) ± long chain n-3 polyunsaturated fatty acids (LC n-3 PUFA) on skeletal muscle mass and strength in older, healthy adults. We used this subset to identify biomarkers of sarcopenia risk (moderate- and high-risk) and also correlate the HDL proteome with hand- and leg-strength and skeletal muscle index (SMI). Using this approach we identified several biomarkers associated with high-sarcopenia risk on small (S) and (L) HDL including ceruloplasmin, prothrombin, actin cytoplasmic 1 and paraoxonase-1. Similarly we identified several proteins that correlated with total handgrip- and leg-strength and SMI and also with changes in these parameter (representing SMI or strength loss/gain). Interesting, there was a unique relationship with each parameter and the HDL proteome, suggesting that different mechanisms can lead to muscle/strength loss. Additionally in this study, we investigated the impact of supplementation on the HDL proteome. We showed that the HDL proteome is uniquely affected by even small changes in diet (Leu ± LC n-3 PUFA) and may reflect positive changes in patients that are not yet seen in more physical measures such as muscle mass and strength.

In study 2, we investigated the utility of the HDL proteome as a biomarker of cachexia and sarcopenia (low muscle mass) in patients with gastrointestinal cancer. In patients with cachexia, we identified *n*=16 biomarkers including vitronectin, beta-ala-his dipeptidase, apolipoprotein A-II and ceruloplasmin, when comparing patients to those without cachexia. A score was created using all the identified proteins and its performance was evaluated using receiver operating characteristic (ROC) area under the curve (AUC). The score had an AUC of 0.810, which is considered clinically relevant, and performed better than individual proteins or the commonly proposed cachexia screening tool, the modified

Glasgow prognostic score (mGPS) (AUC=0.536). A similar approach was used to identify a sarcopenia score in this group. We identified *n*=13 biomarkers associated with sarcopenia including beta-ala-his-dipeptidase, sun domain containing protein 3, selenoprotein P and alpha 2 macroglobulin. Significant proteins were combined into a score and its AUC was 0.861.

In study 3, we supported our findings in a C26 model of cachexia. The HDL of mice with cachexia (C26) was significantly different from mice without cachexia (NC) and control mice (CT), while there were few differences between the NC and CT mice. Similarly, the livers of C26 mice were significantly different from NC and CT mice, with upregulated pathways related to protein synthesis, downregulation of pathways related to xenobiotic metabolism and activation of the acute phase response. This study also allowed us to substantiate several biomarkers identified in the human cohort including apolipoprotein B, ceruloplasmin, hepatocyte growth factor activator, 78kDa glucose-regulated protein, Insulin growth factor binding protein 3, vitamin K dependent protein C, alpha-1-antichymotrypsin, thyroxine binding globulin and vitronectin.

To conclude, in this thesis, we have shown that the HDL proteome is a potentially robust biomarker of cancer cachexia. Further research is needed to validate these findings first in a cohort of similar cancer patients and then in a more diverse cohort. We have also shown that the liver of mice with cachexia is severely affected, with increased protein synthesis and reduced xenobiotic metabolism, contributing to the scarce research on the subject.

Contents

Chapter 1:

Circulating Biomarkers of Cachexia and Sarcopenia in Gastro-intestinal cancer patients: A Rapid Review

Circulating Biomarkers of Cachexia and Sarcopenia in Gastro-intestinal cancer patients: A Rapid Review

Rianna McElroy[1], Erin S. Sullivan[2,3,4], Kathleen A. J. Mitchelson[1], Aoife Ryan[2,3], Fiona C. McGillicuddy[5], Helen M. Roche[1,6]

1. School of Public Health, Physiotherapy and Sport Science, Conway Institute, Institute of Food and Health, University College Dublin , Dublin, Ireland,

2. School of Food & Nutritional Sciences, College of Science, Engineering & Food Science, University College Cork, Cork, Republic of Ireland,

3. Cork Cancer Research Centre, University College Cork, Cork, Republic of Ireland,

4. Writing Group, The European Union Thematic Network on Integrated Nutrition in Cancer Care (INC2), Croydon, UK

5. Diabetes Complications Research Centre, Conway Institute and School of Medicine, University College Dublin, Dublin 4, Republic of Ireland,

5. Institute for Global Food Security, Queen's University Belfast, Belfast, UK.

*Corresponding Author 1: Professor Helen M. Roche

Address: UCD Conway Institute, University College Dublin, Belfield, Dublin D04 H1W8,

Ireland

Email: helen.roche@ucd.ie

Telephone: +353 1 716 6845

#Corresponding Author 2: Assistant Professor Fiona C. McGillicuddy

Address: UCD Conway Institute, University College Dublin, Belfield, Dublin D04 H1W8,

Ireland

Email: fiona.mcgillicuddy@ucd.ie

Telephone: +353 1 716 6769

Chapter prepared as manuscript, Journal to be decided.

Abstract:

Introduction: Cancer cachexia is a debilitating wasting condition characterised by rapid weight loss that can occur in up to 80% of cancer patients. It is typically defined as weight loss >5% in 6 months or 2% in patients with low BMI or low muscle mass (sarcopenia). It leads to reduced survival and quality of life and increased treatment complications, however, despite the seriousness of the disease, it is vastly underdiagnosed. Sarcopenia can also occur in cancer patients and may be age or tumour associated. It leads to reduced survival and poor outcomes and like cachexia, is underdiagnosed in the clinic. Lack of a standardised definition and cut-off points, difficulties in conducting assessment and lack of awareness all contribute to this underdiagnosis. A blood based biomarker that can be integrated into routine care would greatly improve the diagnosis of these conditions, however to date none have made it to clinic. In this rapid review we aimed to summarise from the literature circulating biomarkers of cachexia and sarcopenia in patients with gastrointestinal cancer.

Methods: A search-strategy was developed using key-words related to cachexia, sarcopenia, gastro-intestinal cancers (gastric, colorectal and oesophageal) and circulating mediums e.g. blood, serum, plasma. The refined search was conducted in *n*=2 databases, Embase and PubMed. Retrieved papers were uploaded to Rayyan and duplicates removed prior to screening. Titles and abstracts were screened using the inclusion criteria before full-text review of selected papers. An additional search of references, important journals and key authors in the field was conducted. Data extraction and synthesis was carried out on selected papers.

Results: In total, *n*=1860 papers were identified after duplications were removed. After screening of title, abstract and full-texts, *n*=67 papers met the inclusion criteria and an additional *n*=6 papers were included from other sources (references, journals, authors), totalling *n*=73 papers. Of these, *n*=37 papers identified biomarkers of cachexia and *n*=36 papers identified biomarkers of sarcopenia. In total, *n*=46 biomarkers were identified for cachexia including *n*=21 inflammatory markers (CRP, albumin, IL-6 etc.), *n*=7 metabolites and nutrition related markers (haemoglobin, haematocrit, total protein, bile acids etc.), *n*=8 hormones (adiponectin, ghrelin, insulin, IGF-1 etc.), *n*=3 lipid profile related markers (HDL, LDL, triglycerides) and *n*=5 tumour and other markers (Immunosuppressive acidic, Carcinoembryonic antigen, IGFBP3 etc). For sarcopenia, *n*=31 potential biomarkers were identified including, *n*=20 inflammatory markers (CRP, albumin, mGPS, NLR etc.), *n*=5 metabolites and nutrition related markers (haemoglobin, total protein, calcium etc.), *n*=2 hormones (Irisin and FGF21), and *n*=4 tumour and other markers (Immunosuppressive acidic, creatine kinase,MiR-203 and Cholinesterase).

Conclusion: Inflammatory markers, including acute phase proteins, cytokines and cell counts, were the most abundant biomarker type identified in the literature. The most commonly identified biomarker

of cachexia and sarcopenia were the routine laboratory markers CRP, albumin and haemoglobin. Many of the biomarkers face challenges such as reproducibility and specificity and so have not been integrated into routine care for the detection of these disorders. More research is necessary to identify a suitable biomarker.

Introduction

Cancer cachexia is a metabolic wasting disorder that manifests in certain patients during cancer development[1]. It is characterised primarily by rapid body weight loss[2], due to skeletal muscle with or without adipose tissue or fat loss, associated with other hallmarks including inflammation[3], anorexia[4], insulin resistance[5] and disturbances in muscle anabolic and catabolic pathways[6]. Prevalence varies across cancer type, with gastrointestinal cancers having the highest incident rates and breast, skin and haematological having some of the lowest[7, 8]. Similarly, sarcopenia is a wasting disorder characterised by muscle atrophy, with reduced strength and functionality[9]. Typically, sarcopenia is defined as strength and muscle loss associated with old age[9], however the term is frequently used in the cancer setting to refer to low muscle mass[10]. As the majority of people with cancer are older, they are already at an increased risk of sarcopenia[11-13]. However as muscle mass is not frequently evaluated prior to diagnosis, it is impossible to tell if muscle loss is age or cancer dependent. The mechanisms contributing to age-related sarcopenia include low protein intake[14] and physical activity[15, 16], hormonal changes[17], and "inflammaging"[18]. Unlike cachexia, it is associated with reduced energy expenditure[19] and adipose tissue accumulation[20, 21]. The prevalence of sarcopenia was reported to be 33% in a pan-cancer study of *n*=13761 patients, with highest prevalence in gastrointestinal cancers and lowest in breast cancer, mimicking cachexia[19]. This is compared to a prevalence of 10-27% in the general population[22].

Cachexia and sarcopenia are detrimental to patients with cancer, as both lead to treatment related toxicity[23-25], surgical complications[26-29] and reduced survival[30-32]. However, despite their significant impact on patient outcomes, the diagnosis of cachexia and sarcopenia in cancer patients remains challenging. One of the biggest challenges facing the identification of cachexia and sarcopenia is the plethora of definitions, criteria and cut-off points available. Indeed, in a survey of *n*=2375 health care practitioners (doctors (32.8%), nurses (13.6%), dietitians (27.7%), pharmacists (12.4%) and physical therapists (7.1%)), 43.6% of respondents said clinicians do not screen for cachexia because "They do not know how to effectively screen patients" and 38.8% said "There are no standardized tools or instruments to screen patients for cachexia"[33]. Additionally, only 29.1% of respondents correctly identified weight loss >5% as a criteria for cachexia[33]. Currently, the Fearon et al., definition is the most widely accepted classification of cancer cachexia, reached by consensus in 2011[2]. It defines cachexia as weight loss greater >5% in the previous 6 months, or weight loss >2% in individuals with a BMI <20 kg/m^2 or reduced skeletal muscle mass (sarcopenia)[2]. Other definitions such as the Evans et al.,[34] and screening tools such as the Global Leadership Initiative on Malnutrition (GLIM)[35] combine weight loss

and aetiological criteria (for example reduced food intake, high C-reactive protein (CRP), fatigue) for the diagnosis of cachexia[34, 35].

The diagnosis of sarcopenia in cancer is equally as challenging. Various diagnostic frameworks, such as those proposed by the European Working Group on Sarcopenia in Older People 2 (EWGSOP2), which uses muscle strength as the primary diagnostic measurement[36], and the Asian Working Group for Sarcopenia (AWGS)[37], are used in the general population, however, these are not specifically tailored for use in patients with cancer and therefore may not be applicable. In the cancer setting, several skeletal muscle cutoff points are suggested, as summarised by Ryan et al[38]., including the Prado et al.,[39] Baumgartner et al.,[40] Martin et al.,[31] and Fearon et al.,[2, 38] cut-off points[37]. The differences in prevalence by definition/cut-off point highlights the severity of this issue in the clinical setting, putting many patients at risk of not being diagnosed[41, 42].

Also contributing to the diagnostic challenges are the methods of measurement. The diagnosis of cachexia for example often relies on patients knowing their weight from 6 months prior[2]. Body weight can also be a blunt tool in a population at risk of water retention[43] and in patients with obesity where weight loss may be less noticeable or considered less concerning[44]. Muscle wasting can also be hidden in obesity and so body composition measurements are crucial to identify those with sarcopenia[44]. Computed Tomography (CT) scans and Magnetic Resonance Imaging (MRI) are considered gold standard for muscle quantification[45], with the lumbar 3rd vertebra being a common location for imaging in cancer patients[46]. While this provides highly accurate muscle quantification, not all cancer patients receive a CT scan of this area if their tumour is located elsewhere[47]. CT and MRI are also expensive, time consuming and require highly trained staff[48] and in the case of CT scans, expose the patient to radiation (2 millisieverts (mSv)-31 mSv)[49]. These issues make CT scans and MRI an unfeasible option for repeated measures and tracking changes in patients over time. Other options include dual-energy X-ray absorptiometry (DXA) and bioelectrical impedance analysis (BIA)[50]. DXA is used to quantify appendicular skeletal muscle mass[36]. It is a fast, reliable and accessible method, however, it is imperative the same machine is used for repeat measurement, as there is a high degree of variability between machines[51]. It also requires trained staff and can be affected by the hydration levels of the patient[36]. Although small, DXA also exposes the patient to low dose radiation (~0.5 µSv)[51] which may be unfavourable for repeated measurement. BIA is also a fast and reliable method in the clinical setting[52]. It does not measure muscle mass directly but uses electric currents to determine the level of resistance (different tissues have different resistance) and estimates body composition based on a reference cohort of DXA scans[36]. Therefore it is necessary that the reference population reflects the patient population being investigated. While screening tools have been developed for the detection of malnutrition and sarcopenia such as the Strength, Assistance with walking, Rise from a chair, Climb

stairs, and Falls (SARC-F)[53], Malnutrition Screening Tool (MST)[54], Patient-Generated Subjective Global Assessment (PG-SGA)[55, 56], these tests also require time, staff and sometimes body weight recall to complete the assessments. Additionally, Ní Bhuachalla et al., evaluated the MUST and MST tools in *n*=725 cancer patients and found that they misdiagnosed 27% and 35% respectively of patients with cachexia and 55% and 61% respectively of patients with sarcopenia[57]. However the Nutritional Risk Index, which uses body weight loss and serum albumin to make a score only misdiagnosed 7% of patients with cachexia as low risk for malnutrition, while it misdiagnosed only 14% of patients with sarcopenia, highlighting the advantage of blood based markers for diagnosis[57].

The challenges highlighted here are reflected in patient experiences. A survey of *n*=1,073 Irish patients with cancer by Sullivan et al., found that while patients were weighed often, dietary changes were rarely addressed and only 39% of weight losing patients were seen by a registered dietitian. This was despite high rates of weight loss (44%) and patient reported muscle loss (52%)[58]. Another study reported patients desired greater acknowledgment of their weight loss from care providers[59]. With these challenges in mind, a blood based biomarker for cachexia and sarcopenia, that can be introduced into routine care, is paramount in the cancer setting. It would provide clear, actionable markers and allow for continuous monitoring during treatment in a non-invasive, cost effective manner. Such a biomarker could bridge the gap between patient reported symptoms and clinical intervention, ensuring that muscle loss and weight changes are addressed proactively, ultimately improving patient outcomes and quality of care.

While many biomarkers have been suggested in the literature, to date none have made it to clinic. Inflammatory markers have been particularly popular in the cachexia space due to the inflammatory nature of the disease[60], while other markers have included wasting products (fatty acids)[61, 62] and also tumour derived cachexia inducers (for example, Insulin growth factor binding protein 3 (IGFBP3)[63]. Inflammatory markers such as C-reactive protein (CRP)[64, 65], Interleukin-6 (IL-6)[65, 66] and Tumour necrosis factor-alpha (TNFα)[67-69] have previously been identified as cachexia biomarkers, however they lack specificity. This is particularly the case for CRP, as this acute phase protein derived from the liver is used as a broad spectrum inflammatory marker[70]. TNFα or cachexin, is one of the original biomarkers/mediators of cachexia, but it has received less attention in recent years due to its ubiquitous functionality. Furthermore, it has a short half-life[71], low bioavailability[71] and can be profoundly affected by anti-coagulants in blood tubes[72] and the method of measurement[73], making it a less desirable biomarker. Additionally, TNFα has not been consistently identified as a biomarker of cachexia. A recent systematic review by Paval et al., including *n*=1,277 patients found that although TNFα was significantly different from healthy controls, it was not consistently different from non-weight losing cancer patients[74]. Growth/differentiating factor 15 (GDF15), also known as Macrophage

in various cancer types (lung and pancreatic[75], gastrointestinal, genito-urinary head and neck, etc[76]) and is associated primarily with anorexia[77], mediated through the glial cell-derived neurotrophic factor receptor α-like (GFRAL) in the brain[78, 79]. However, it is important to note that MIC-1/GDF15, is upregulated in response to ageing[80], and smoking[81], factors which could affect its status as a cachexia biomarker. Additionally, increased MIC-1/GDF15 has not been consistently reported as a biomarker of cachexia. MIC-1 was upregulated in patients with lung but not pancreatic cancer in one study[82] while in another study of patients with lung and gastrointestinal cancer, it was associated with anorexia but not weight loss or low SMI[77].

In this rapid review, we hoped to take a systematic approach to further explore and summarise potential circulating biomarkers of cancer cachexia and sarcopenia in gastrointestinal cancer. We focused our attention on oesophageal, gastric and colorectal cancer to closely mimic our cohort of patients, used for biomarker discovery. We focused on circulating biomarkers as they are less invasive for the patient and most likely to be integrated into routine care. We separately analysed biomarkers of cachexia and sarcopenia to identify distinct markers for each condition that could help differentiate and stratify them. This review will provide an informative basis for understanding the current landscape of circulating biomarkers in cancer cachexia and sarcopenia, offering insights that could guide future research and clinical practice aimed at improving diagnosis, treatment, and patient outcomes in gastrointestinal cancer

Methods

Inclusion/exclusion criteria:

Papers were considered for inclusion if they 1. were original research articles, 2. published within 15 years of the search date (27th of November 2023), 3. the study population consisted of patients with gastric, oesophageal or colorectal cancer, 4. was conducted in adults over 18 years of age, 5. in patients with cachexia (weight loss) or sarcopenia (low muscle mass) by any definition, 6. measured potential biomarkers in circulation using blood, serum or plasma and 7. were published in English. Biomarker discovery did not have to be the main outcome for inclusion.

Search strategy:

A search strategy was developed starting with keywords related to cachexia and sarcopenia, including “malnutrition”, “wasting” and “body composition”. Keywords related to our outcome of interest, biomarker discovery, were included such as “mediators”, “signature” and “profile”. The names of potential biomarkers were also included in the search, to capture papers whose primary outcome was not biomarker discovery, including “IL-6”, “CRP” and “albumin”, which have previously been identified as cachexia biomarkers. Keywords related to cachexia and sarcopenia were restricted to title and abstract to prevent the inclusion of unrelated papers. The search was first conduced in PubMed before being adapted for Embase. The search was expanded until the resulting number of papers did not increase drastically and there did not appear to be a large number of unrelated papers. Table 1 includes the search strategy development. The final search was conducted on the 27th of November 2023. An additional search was conducted by the reviewer to identify potential papers missed by the database search. This included scanning references of the selected papers, searching published work of well-known authors in the field (J.M Argilés V.E Baracos, S.M Anker, K.C.H Fearon, T.A Zimmers, RJ Skipworth) and searching key journals (Journal of Cachexia, Sarcopenia and Muscle. Nature Cancer).

Table 1: Search strategy development. Search was originally developed for PubMed before adapting to Embase.

Order of search	Search terms	Number of articles found
1	((Cachexia OR Sarcopenia OR "low muscle mass" OR "muscle mass" OR "weight loss" OR Wasting OR "low skeletal muscle index") AND ("gastroesophageal cancer" OR "gastric cancer" OR "colorectal cancer" OR "colon cancer" OR "oesophageal cancer" OR "esophageal cancer" OR "gastric junction cancer" OR "stomach cancer" OR "bowel cancer" OR "throat cancer")) AND (Biomarker OR marker OR profile OR signature OR molecular OR molecule OR pattern) AND ((blood OR serum OR plasma OR circulating OR circulation) AND (Protein OR	149

	proteomics OR metabolite OR DNA OR RNA OR MicroRNA OR inflammatory OR cytokine OR chemokine OR cell))	
2	((Cachexia OR Sarcopenia OR "low muscle mass" OR "muscle mass" OR "weight loss" OR Wasting OR "low skeletal muscle index") AND ((cancer[MeSH]) AND (gastroesophageal OR gastric cancer OR colorectal OR colon OR oesophageal OR esophageal OR “gastric junction” OR stomach cancer OR bowel OR throat))) AND (Biomarker OR marker OR profile OR signature OR molecular OR molecule OR pattern OR Protein OR proteomics OR metabolite OR DNA OR RNA OR MicroRNA OR inflammatory OR cytokine OR chemokine OR cell)	1563
3	((Cachexia[tiab] OR Sarcopenia[tiab] OR "low muscle mass"[tiab] OR "muscle mass"[tiab] OR "weight loss"[tiab] OR Wasting[tiab] OR "low skeletal muscle"[tiab]) AND ((cancer[MeSH]) AND (gastroesophageal OR gastric OR colorectal OR colon OR oesophageal OR esophageal OR “gastric junction” OR stomach OR bowel OR throat))) AND (Biomarker OR marker OR profile OR signature OR molecular OR molecule OR pattern OR Protein OR proteomics OR metabolite OR DNA OR RNA OR MicroRNA OR inflammatory OR cytokine OR chemokine OR cell OR associated OR predict) NOT Review	1257
4	((Cachexia[tiab] OR Sarcopenia[tiab] OR "low muscle mass"[tiab] OR "muscle mass"[tiab] OR "weight loss"[tiab] OR Wasting[tiab] OR "low skeletal muscle"[tiab]) AND ((cancer[MeSH]) AND (gastroesophageal OR gastric OR colorectal OR colon OR oesophageal OR esophageal OR “gastric junction” OR stomach OR bowel OR throat))) AND (Biomarker OR marker OR profile OR signature OR molecular OR molecule OR pattern OR Protein OR proteomics OR metabolite OR DNA OR RNA OR MicroRNA OR inflammatory OR cytokine OR chemokine OR cell OR CRP OR il-1 OR il-6 OR Activin-A OR MURF-1 OR TIMP-1 OR insulin OR ghrelin OR Serpin OR STAT3 OR TGFβ OR GDF15 OR ZAG OR FGF21 OR hormone OR associated OR predict) NOT Review NOT chemotherapy	1253
5	((Cachexia[tiab] OR “cancer cachexia” OR malnutrition OR Sarcopenia[tiab] OR "low muscle mass"[tiab] OR "muscle mass"[tiab] OR "weight loss"[tiab] OR Wasting[tiab] OR "skeletal muscle"[tiab]) AND ((cancer[MeSH]) AND (gastroesophageal OR gastric OR colorectal OR colon OR oesophageal OR esophageal OR “gastric junction” OR stomach OR bowel OR throat))) AND (Biomarker OR marker OR profile OR signature OR molecular OR molecule OR mediators OR serological OR pattern OR factor* OR Protein OR proteomics OR metabol* OR DNA OR RNA OR MicroRNA OR inflammatory OR cytokine OR chemokine OR circulating OR cell OR CRP OR IL-1 OR IL-6 interleukin OR Activin-A OR MURF-1 OR TIMP-1 OR insulin OR ghrelin OR Serpin OR STAT3 OR TGF-β OR TGF-α OR myostatin OR GDF15 OR ZAG OR FGF21 OR albumin OR fatty acids OR lipids OR hormone OR associated OR predict) NOT Review NOT chemotherapy NOT outcomes	1051
6	((Cachexia[tiab] OR “cancer cachexia” OR malnutrition OR Sarcopenia[tiab] OR "low muscle mass"[tiab] OR "muscle mass"[tiab] OR "weight loss"[tiab] OR Wasting[tiab] OR "skeletal muscle"[tiab]) AND ((cancer[MeSH]) AND (gastroesophageal OR gastric OR colorectal OR colon OR oesophageal OR esophageal OR “gastric junction” OR stomach OR bowel OR throat))) AND (Biomarker OR marker OR profile OR signature OR molecular OR molecule OR mediators OR serological OR pattern OR factor* OR Protein OR proteomics OR metabol* OR DNA OR RNA OR MicroRNA OR inflammatory OR cytokine OR chemokine OR circulating OR cell OR CRP OR IL-1 OR IL-6 interleukin OR Activin-A OR MURF-1 OR TIMP-1 OR insulin OR ghrelin OR leptin OR “extracellular vesicles” OR Serpin OR STAT3 OR TGF-β OR TGF-α OR myostatin OR GDF15 OR ZAG OR FGF21 OR albumin OR fatty acids OR lipids OR hormone OR associated OR predict*) NOT Review NOT chemotherapy NOT outcomes	1095
7	((Cachexia[tiab] OR "cancer cachexia" OR malnutrition OR Sarcopenia[tiab] OR "low muscle mass"[tiab] OR "muscle mass"[tiab] OR "weight loss"[tiab] OR Wasting[tiab] OR "skeletal muscle"[tiab] OR "body composition") AND ((cancer[MeSH]) AND (gastroesophageal OR gastric OR colorectal OR colon OR	1162

	oesophageal OR esophageal OR "gastric junction" OR stomach OR bowel OR throat))) AND (Biomarker OR marker OR profile OR signature OR molecular OR molecule OR mediators OR serological OR pattern OR factor* OR Protein OR proteomics OR metabol* OR DNA OR RNA OR MicroRNA OR inflammatory OR cytokine OR "acute phase" OR chemokine OR circulating OR cell OR CRP OR IL-1 OR IL-6 interleukin OR Activin-A OR MURF-1 OR TIMP-1 OR insulin OR ghrelin OR leptin OR "extracellular vesicles" OR Serpin OR STAT3 OR TGF-β OR TGF-α OR myostatin OR GDF15 OR ZAG OR FGF21 OR albumin OR "fatty acid*" OR lipids OR hormone OR associated OR predict*) NOT Review NOT chemotherapy NOT outcomes	
8	((Cachexia[tiab] OR "cancer cachexia" OR malnutrition OR Sarcopenia[tiab] OR "low muscle mass"[tiab] OR "muscle mass"[tiab] OR "weight loss"[tiab] OR Wasting[tiab] OR "skeletal muscle"[tiab] OR "body composition") AND ((cancer[MeSH]) AND (gastroesophageal OR gastric OR colorectal OR colon OR oesophageal OR esophageal OR "gastric junction" OR stomach OR bowel OR throat))) AND (Biomarker OR marker OR profile OR signature OR molecular OR molecule OR mediators OR serological OR Serum OR Plasma OR pattern OR factor* OR Protein OR proteomics OR metabol* OR DNA OR RNA OR MicroRNA OR inflammatory OR cytokine OR "acute phase" OR chemokine OR circulating OR cell OR CRP OR IL-1 OR IL-6 interleukin OR Activin-A OR MURF-1 OR TIMP-1 OR insulin OR ghrelin OR leptin OR "extracellular vesicles" OR Serpin OR STAT3 OR TGF-β OR TGF-α OR myostatin OR GDF15 OR ZAG OR FGF21 OR albumin OR "fatty acid*" OR lipids OR hormone OR associated OR predict*) NOT Review NOT chemotherapy NOT outcomes	1166

Screening:

Papers identified in the search were uploaded to Rayyan and duplicates removed. Screening of the title and abstract was conducted using the inclusion criteria before full-text screening of selected papers. Screening was conducted by *n*=1 reviewer (R. McElroy)

Data extraction:

Data extracted from the studies included location, number of participants, cancer type, average age of cohort, treatment status, method of body composition/weight measurement (e.g self reported, measured in clinic), the source of the biomarker (e.g serum/plasma), biomarker recorded and definition used for diagnosing cachexia or sarcopenia. The data extraction was carried out by one reviewer (R. McElroy) and the final table checked by another (E. Sullivan).

Quality assessment:

In line with acceptable rapid review shortcuts[83], quality assessment was not carried out on selected papers.

Results

Results of paper screening:

Using the final search strategy outlined in table 1, *n*=1166 papers were identified in PubMed and *n*=694 papers were identified in Embase, totalling *n*=1860 papers (Figure 1). After uploading to Rayyan, *n*=306 duplicates were removed. Using the inclusion criteria, the title and abstract were reviewed resulting in *n*=1407 excluded papers, *n*=108 accepted papers and *n*=39 maybe papers. A full text screening of maybe papers was carried out to determine if they should be included, resulting in *n*=1440 excluded papers and *n*=114 accepted papers. Following this, a full text review resulted in the inclusion of *n*=67 papers and exclusion of *n*=47 papers. The additional search of references, key authors and journals resulted in the inclusion of *n*=6 papers. Therefore, *n*=73 papers were selected for this study (Supplementary Tables 1 & 2). Papers were excluded for a variety of reasons including, the potential biomarker not being measured in circulation, patients not characterised by weight loss or skeletal muscle mass and wrong cancer type. Access was requested to one paper however this was not granted and therefore the paper was excluded.

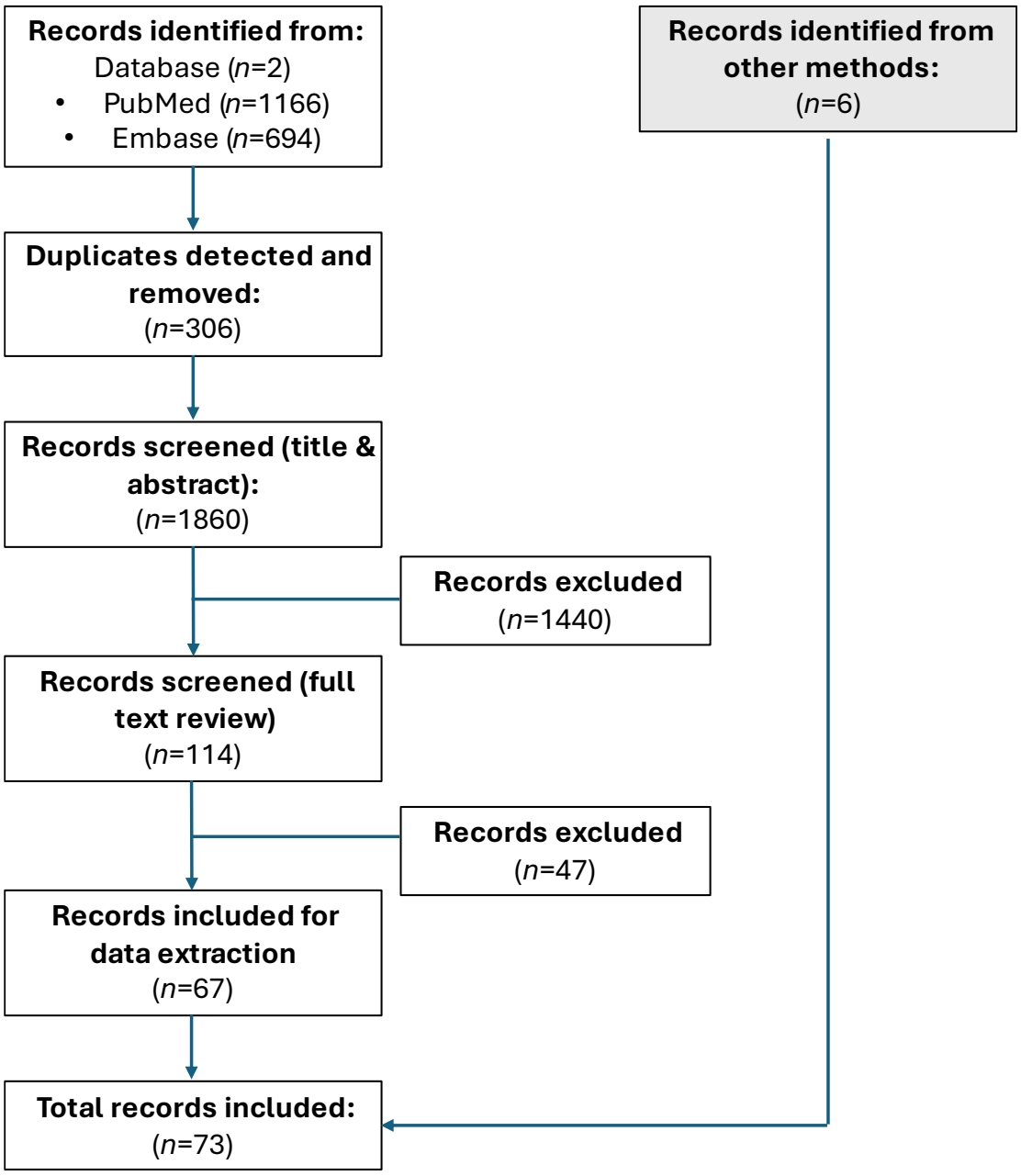

Figure 1: Flow chart of search, paper screening and selection

General characteristics of included studies:

The majority of the studies were conducted in patients with colorectal cancer (*n*=32), followed by gastric (*n*=16) and oesophageal (*n*=13) while the remaining (*n*=12) were a mix of the three. The majority of studies measured biomarkers in serum (*n*=51), while plasma (*n*=5) and whole blood (*n*=7) were also used. The remaining (*n*=10) used more then one medium in their study. Men and women were included in all studies, except for *n*=1 which only included men. The average age of patients ranged from late 50's to 70's. Interestingly, *n*=35 studies were conducted in patients who were pre-treatment (chemotherapy/radiation/surgery), *n*=26 were conducted in patients who were pre-surgery, *n*=5 were conducted in patients who had not received treatment in the 4 weeks prior to assessment and the remaining *n*=7 had participants at different stages of treatment, had all received treatment or treatment status was unclear while *n*=1 study looked at biomarkers before and after chemotherapy. Of the papers included, *n*=37 investigated cachexia, *n*=35 sarcopenia and *n*=1 pre-sarcopenia, the results of which are included with the sarcopenia data. Within the papers classified as cachexia related, *n*=3 investigated "malnutrition". To classify malnutrition, *n*=1 study[84] used the Global Leadership Initiative on Malnutrition (GLIM) criteria and n=1 study[85] used the Mini Nutritional Assessment (MNA)[85]. Of the studies included, *n*=40 were conducted in Asia, *n*=16 in Europe, *n*=4 in South America, *n*=3 in North America and *n*=1 in Eastern Europe. Also included were *n*=5 studies which did not explicitly state where the study took place.

Cachexia

Papers investigating cachexia and sarcopenia were analysed separately to determine biomarkers representative of each condition. Various definitions of cachexia were used in the included studies (Supplementary Table 1). Only *n*=4 studies used the Fearon et al.,[2] criteria to define cachexia while *n*=3 used the Evans et al., definition[34]. Other studies used weight loss alone to define cachexia (*n*=5 used >5% weight loss in 3 months) while others used weight loss and etiological criteria such as CRP >10mg/L (*n*=3 studies used >10% weight loss and CRP >10mg/L). Additionally, *n*=5 studies investigated weight loss of any amount. Prevalence of cachexia ranged from 23.17%-74.42% using the studies chosen definition.

Biomarkers:

In the included studies, *n*=46 potential biomarkers were identified. These were classified by their most relevant function into lipid profile related biomarkers, inflammatory biomarkers, metabolites and nutrition related biomarkers, hormones and tumour and other markers. Cell based measurements (neutrophils, lymphocytes, leucocytes and their ratios) were included with the inflammatory markers as they are frequently used as markers of systemic inflammation, particularly the

neutrophil/lymphocyte ratio[86-88]. Tables are organised in descending order of biomarkers most commonly identified in the literature.

Inflammatory markers:

From the included studies, *n*=21 inflammatory markers were identified including cytokines (Table 2) (Interleukin (IL)-6, IL-8, IL-10, tumour necrosis factor alpha (TNFα) and interferon alpha (INFα)), acute phase proteins (C-reactive protein (CRP), albumin, haptoglobin, alpha-1-antichymotrypsin (SERPINA3) and fibrinogen) and blood cell counts and associated ratios (total lymphocyte count, neutrophil-lymphocyte ratio (NLR), leukocyte-lymphocyte ratio (leu-lymph ratio), among others. Albumin (*n*=11) and CRP/high sensitivity (HS)-CRP (*n*=13) were identified as significantly different in the greatest number of studies, likely due to their routine measurement in clinic, followed by IL-6 (*n*=8) and IL-8 (*n*=7).

CRP/HS-CRP was consistently upregulated in CC compared to WSC in all studies, identifying it as a potential biomarker (*n*=13). Concentrations of CRP ranged from 3.23mg/L to 39 mg/L in WSC and 4.62mg/L to 82.7mg/L in CC. Deans et al., found that weight loss correlated with CRP ($r=0.24$, $p<0.001$)[89] while another study from the same group found that CRP was a good predictor of weight loss using receiver operating characteristics (ROC) area under the curve (AUC) (AUC=0.72, $p<0.001$)[90]. Also included were *n*=3 studies that used HS-CRP. Concentrations of HS-CRP ranged from 1.9 mg/L to 46mg/L in WSC while it ranged from 5.4mg/L to 107mg/L in CC. In a study that categorized patients by HS-CRP levels into <1 mg/L and ≥1 mg/L, it was found that weight loss greater than 2.4% and a BMI below 18.5 were linked to HS-CRP levels of ≥1 mg/L[91].

Albumin was consistently downregulated in CC compared to WSC (*n*=11). Concentrations ranged from 34 g/L to 45.54g/L in WSC and 25g/L to 39.78g/L in CC. Albumin correlated with weight loss in *n*=1 study ($r=-0.253$, $p<0.001$)[92] and was significantly decreased in late but not early stage CC in another study[93].

The combination of CRP and albumin into various score has also been identified as a potential biomarker of cachexia. The modified Glasgow prognostic score (which uses cutoff values of >10mg/L CRP and <35g/L albumin to make a score) was significantly associated with weight loss in patients with gastroesophageal adenocarcinoma ($p=0.002$)[94]. In another study, there were significantly more CC patients with a high mGPS score compared to WSC ($p<0.001$), and similarly, the CRP to albumin ratio was significantly different between WSC and CC[95].

The cytokine IL-6 (*n*=8) was consistently upregulated in CC in the included studies. Concentrations ranged from 0.83 pg/ml to 12.37 pg/ml in WSC and 2.1 pg/ml to 65.22 pg/ml in CC. Il-6 in n=2 studies

increased progressively with disease severity, with lower levels found in early versus late stage cachexia (7.2 ± 4.3 pg/ml and 13.3 ± 7.4 pg/ml respectively)[96] and in those at risk of malnutrition versus malnourished patients (13.5 ± 1.8 pg/ml and 25.1 ± 2.5 pg/ml)[85].

TNFα, or cachexin, was upregulated in CC in the studies included (n=4). Concentrations of the protein ranged from 4.77 pg/ml to 8 pg/ml in WSC and 6 pg/ml to 19.1 pg/ml in CC. TNFα increased progressively with disease severity, with lower levels found in early versus late stage cachexia (10.2 ± 5.0 pg/ml and 16.7 ± 6.7 pg/ml respectively)[96] and in those at risk of malnutrition versus malnourished patients (9.4 ± 1.0 pg/ml and 19.1 ± 1.3 pg/ml)[85].

The acute phase proteins SERPINA3 and haptoglobin were associated with weight loss in *n*=1 study of patients with gastro-oesophageal[90], while fibrinogen was upregulated in patients experiencing weight loss (p<0.001) in *n*=1 study[97]. Transferrin was downregulated in CC in n=4 studies and ranged from 233 mg/dl to 367 mg/dl in WSC and 189 mg/dl to 314 mg/dl in CC. In *n*=1 study, transferrin was not significantly downregulated in CC compared to WSC (231 mg/dl and 250.08 mg/dl respectively) however using ROC analysis, it was classified as a good predictor of weight loss with an optimal cut-off value of > 206mg/dl and a specificity of 82.4% and sensitivity of 34.1%[98].

Macrophage inhibitory cytokine 1 (MIC-1), or Growth/differentiation factor 15 (GDF15), was identified as a biomarker of cachexia in *n*=1 study. The protein did not correlate with weight loss in the study but levels were higher in those with CC (1493 pg ml^{-1}) compared to WSC (1256 pg ml^{-1}) (p=0.036)[99].

Table 2: Inflammatory markers identified in cachexia

Inflammatory markers	
CRP/HS-CRP* (*n*=13)	• Acute-phase response proteins are related to cachexia and accelerated angiogenesis in gastroesophageal cancers • Adipokines and ghrelin in gastric cancer cachexia • Assessment of nutritional and inflammatory status to determine the prevalence of malnutrition in patients undergoing surgery for colorectal carcinoma • Cancer cachexia induces morphological and inflammatory changes in the intestinal mucosa • Cancer cachexia is associated with the IL10 -1082 gene promoter polymorphism in patients with gastroesophageal malignancy • Elevation of preoperative serum hs-CRP is an independent risk factor for malnutrition in patients with gastric cancer* • Interleukin-10 gene polymorphisms influence susceptibility to cachexia in patients with low-third gastric cancer in a Chinese population • Myokines in treatment-naive patients with cancer-associated cachexia • Prognostic value of systemic inflammation and for patients with colorectal cancer cachexia • The altered tight junctions: An important gateway of bacterial translocation in cachexia patients with advanced gastric cancer • The influence of systemic inflammation, dietary intake and stage of disease on rate of weight loss in patients with gastro-oesophageal cancer • Tumour-derived transforming growth factor-β signalling contributes to fibrosis in patients with cancer cachexia*

	• Weight loss and resting energy expenditure in male patients with newly diagnosed esophageal cancer*
Albumin (*n*=11)	• Assessment of nutritional and inflammatory status to determine the prevalence of malnutrition in patients undergoing surgery for colorectal carcinoma • Association of interleukin-8 gene polymorphism with cachexia from patients with gastric cancer • Association of interleukin-8 with cachexia from patients with low-third gastric cancer • Associations Between Nutritional Parameters and Clinicopathologic Factors in Patients with Gastric Cancer: A Comprehensive Study • Cancer cachexia induces morphological and inflammatory changes in the intestinal mucosa • Interleukin-10 gene polymorphisms influence susceptibility to cachexia in patients with low-third gastric cancer in a Chinese population • Interleukin-6 induces fat loss in cancer cachexia by promoting white adipose tissue lipolysis and browning • Prognostic value of systemic inflammation and for patients with colorectal cancer cachexia • The altered tight junctions: An important gateway of bacterial translocation in cachexia patients with advanced gastric cancer • The relationship between GLIM-malnutrition, post-operative complications and long-term prognosis in elderly patients undergoing colorectal cancer surgery • Weight loss and resting energy expenditure in male patients with newly diagnosed esophageal cancer
IL-6 (n=8)	• Cancer cachexia induces morphological and inflammatory changes in the intestinal mucosa • Myokines in treatment-naive patients with cancer-associated cachexia • Impact of weight loss on circulating IL-1, IL-6, IL-8, TNF-α, VEGF-A, VEGF-C and midkine in gastroesophageal cancer patients • Assessment of nutritional and inflammatory status to determine the prevalence of malnutrition in patients undergoing surgery for colorectal carcinoma • Circulating leptin and inflammatory response in esophageal cancer, esophageal cancer-related cachexia-anorexia syndrome (CAS) and non-malignant CAS of the alimentary tract • Interleukin-6 induces fat loss in cancer cachexia by promoting white adipose tissue lipolysis and browning • Plasma levels of IL-6 and TNF-Î± in patients with esophageal cancer • Inflammation-induced cholestasis in cancer cachexia
IL-8 (n=7)	• Cancer cachexia induces morphological and inflammatory changes in the intestinal mucosa • Myokines in treatment-naive patients with cancer-associated cachexia • Impact of weight loss on circulating IL-1, IL-6, IL-8, TNF-α, VEGF-A, VEGF-C and midkine in gastroesophageal cancer patients • Cancer cachexia is associated with the IL10 -1082 gene promoter polymorphism in patients with gastroesophageal malignancy • Tumour-derived transforming growth factor-β signalling contributes to fibrosis in patients with cancer cachexia • Association of interleukin-8 with cachexia from patients with low-third gastric cancer • Association of interleukin-8 gene polymorphism with cachexia from patients with gastric cancer
Transferrin (n=4)	• Transferrin and Prealbumin Identify Esophageal Cancer Patients with Malnutrition and Poor Prognosis in Patients with Normal Albuminemia: A Cohort Study • Acute-phase response proteins are related to cachexia and accelerated angiogenesis in gastroesophageal cancers • The influence of systemic inflammation, dietary intake and stage of disease on rate of weight loss in patients with gastro-oesophageal cancer • Assessment of nutritional and inflammatory status to determine the prevalence of malnutrition in patients undergoing surgery for colorectal carcinoma
TNF-α (*n*=4)	• Assessment of nutritional and inflammatory status to determine the prevalence of malnutrition in patients undergoing surgery for colorectal carcinoma • Circulating leptin and inflammatory response in esophageal cancer, esophageal cancer-related cachexia-anorexia syndrome (CAS) and non-malignant CAS of the alimentary tract • Interleukin-6 induces fat loss in cancer cachexia by promoting white adipose tissue lipolysis and browning • Plasma levels of IL-6 and TNF-α in patients with esophageal cancer
IL-10 (*n*=3)	• Cancer cachexia is associated with the IL10 -1082 gene promoter polymorphism in patients with gastroesophageal malignancy

	• Interleukin-10 gene polymorphisms influence susceptibility to cachexia in patients with low-third gastric cancer in a Chinese population • Association of interleukin-10 gene polymorphism with cachexia in Chinese patients with gastric cancer
Pre-albumin (*n*=3)	• Adipokines and ghrelin in gastric cancer cachexia • Transferrin and Prealbumin Identify Esophageal Cancer Patients with Malnutrition and Poor Prognosis in Patients with Normal Albuminemia: A Cohort Study • Weight loss and resting energy expenditure in male patients with newly diagnosed esophageal cancer
mGPS (*n*=2)	• Systemic inflammatory biomarkers as prognostic tools in patients with gastroesophageal adenocarcinoma • Prognostic value of systemic inflammation and for patients with colorectal cancer cachexia
Total lymphocyte count (*n*=2)	• Prognostic value of systemic inflammation and for patients with colorectal cancer cachexia • Associations Between Nutritional Parameters and Clinicopathologic Factors in Patients with Gastric Cancer: A Comprehensive Study
NLR (*n*=1)	• Systemic inflammatory biomarkers as prognostic tools in patients with gastroesophageal adenocarcinoma
Leu-Lymph ratio (*n*=1)	• Systemic inflammatory biomarkers as prognostic tools in patients with gastroesophageal adenocarcinoma
Platelet-lymphocyte ratio (*n*=1)	• Systemic inflammatory biomarkers as prognostic tools in patients with gastroesophageal adenocarcinoma
CRP-albumin ratio (*n*=1)	• Prognostic value of systemic inflammation and for patients with colorectal cancer cachexia
CRP + albumin (*n*=1)	• Decreased liver B vitamin-related enzymes as a metabolic hallmark of cancer cachexia
Lymphocyte to CRP ratio (*n*=1)	• Prognostic value of systemic inflammation and for patients with colorectal cancer cachexia
Lymphocyte-CRP score (*n*=1)	• Prognostic value of systemic inflammation and for patients with colorectal cancer cachexia
SIRI (*n*=1)	• Systemic inflammatory biomarkers as prognostic tools in patients with gastroesophageal adenocarcinoma
IFN-α (*n*=1)	• Tumour-derived transforming growth factor-β signalling contributes to fibrosis in patients with cancer cachexia
SERPINA3 (*n*=1)	• The influence of systemic inflammation, dietary intake and stage of disease on rate of weight loss in patients with gastro-oesophageal cancer
Fibrinogen (*n*=1)	• Preoperative serum fibrinogen is an independent prognostic factor in operable esophageal cancer
Haptoglobin (*n*=1)	• The influence of systemic inflammation, dietary intake and stage of disease on rate of weight loss in patients with gastro-oesophageal cancer
MIC-1 (*n*=1)	• Plasma MIC-1 correlates with systemic inflammation but is not an independent determinant of nutritional status or survival in oesophago-gastric cancer
sTNF-r (*n*=1)	• Cancer cachexia is associated with the IL10 -1082 gene promoter polymorphism in patients with gastroesophageal malignancy
G-CSF (*n*=1)	• Tumour-derived transforming growth factor-Î² signalling contributes to fibrosis in patients with cancer cachexia
GM-CSF (*n*=1)	• The influence of systemic inflammation, dietary intake and stage of disease on rate of weight loss in patients with gastro-oesophageal cancer

Metabolites and nutrition related markers:

The metabolite and nutrition related markers in this study include haematocrit, total protein, bile acids, choline, tryptophan, free fatty acids (FFA) selenium and haemoglobin (

Table 3).

Haematocrit was progressively decreased in patients at risk of malnutrition and malnourished patients with colorectal cancer compared to well nourished patients (37.1 ± 1.2%, 34.7 ± 2.2%, and 40.4 ± 9.6% respectively, p=0.004)[85].

Serum total protein concentration was identified as a biomarker in *n*=1 study wherein it negatively correlated with weight loss (r=−0.281, p<0.0001), and was significantly lower in CC vs WSC (*P* = 0.000022)[92].

Total serum bile acids, and also the bile acids glycoursodeoxycholic acid (GUDCA) and taurochenodeoxycholic acid (TCDCA) were significantly increased in CC compared to WSC with colorectal cancer (p<0.05 for all) however only total bile acids and GUDCA positively correlated with weight loss (p<0.05, for both)[100].

Serum FFA were significantly upregulated in CC compared to WSC in *n*=1 study in patients with gastric or colorectal cancer. Interestingly, FFA were higher in early stage CC (0.55 ± 0.25 mmol/l) compared to late stage CC (0.41 ± 0.25 mmol/l) (p<0.05) and only early stage CC was significantly different to WSC (0.32 ± 0.18 mmol/l) (p<0.05)[96].

In *n*=1 metabolomic study conducted in patients with gastric cancer, several metabolites were altered in the blood of CC with advanced cancer compared to WSC with early stage cancer. These include betaine (p=0.0145), Serine (p=0.0135), threonine (p=0.00690), choline ($p=5.32 \times 10^{-5}$), guanidinoacetate (p=0.000254) and tryptophan (1.13×10^{-10}). However, using ROC analysis, Kojima et al., found that a combination of choline and tryptophan was better able to predict CC (AUC=0.8025) than other metabolites and even CRP + albumin[101].

Selenium was negatively correlated with weight loss in a linear regression of colon cancer patients (β=−0.2, p=0.02) with lowest selenium levels seen in patients who lost >25lbs in the previous year[102].

Haemoglobin was identified as a biomarker in n=7 included studies. Concentration of haemoglobin ranged from 12.6 g/dl to 13.93 g/dl in WSC and 11.1 g/dl to 11.91 g/dl in CC. Haemoglobin correlated with weight loss in patients with gastric cancer (r=−0.138, p=0.01)[92].

Table 3: Metabolite and nutrition related markers identified in cachexia

Metabolites and nutrition related markers	
Haemoglobin (*n*=7)	• Associations Between Nutritional Parameters and Clinicopathologic Factors in Patients with Gastric Cancer: A Comprehensive Study • Cancer cachexia induces morphological and inflammatory changes in the intestinal mucosa • Tumour-derived transforming growth factor-β signalling contributes to fibrosis in patients with cancer cachexia • Circulating leptin and inflammatory response in esophageal cancer, esophageal cancer-related cachexia-anorexia syndrome (CAS) and non-malignant CAS of the alimentary tract • Adipokines and ghrelin in gastric cancer cachexia • Myokines in treatment-naive patients with cancer-associated cachexia • The relationship between GLIM-malnutrition, post-operative complications and long-term prognosis in elderly patients undergoing colorectal cancer surgery
Haematocrit (*n=1)*	• Assessment of nutritional and inflammatory status to determine the prevalence of malnutrition in patients undergoing surgery for colorectal carcinoma
Total protein (*n=1)*	• Associations Between Nutritional Parameters and Clinicopathologic Factors in Patients with Gastric Cancer: A Comprehensive Study
Bile acids	• Inflammation-induced cholestasis in cancer cachexia
Choline + tryptophan (*n=1)*	• Decreased liver B vitamin-related enzymes as a metabolic hallmark of cancer cachexia
Free fatty acids (*n=1)*	• Interleukin-6 induces fat loss in cancer cachexia by promoting white adipose tissue lipolysis and browning
Selenium (*n=1)*	• Selenium, folate, and colon cancer

Hormones:

In the included studies, *n*=8 hormones were identified as biomarkers in cachexia (Table 4). The hunger hormone ghrelin was identified as increased in CC compared to WSC in gastric cancer (2305 ± 818 ng/ml and 1980 ± 913 ng/ml respectively, p<0.001) and negatively correlated with 6 month BMI loss (r=-0.439, p=0.008)[103].

In the same study, IGF-1 was significantly decreased in CC compared to WSC (43.8 ± 9.5 pg/ml and 63.1 ± 13.1 pg/ml, p<0.001) and also insulin (18.4 ± 6.0 µIU/mL and 24.4 ± 6.3 µIU/mL respectively, p=0.04). IGF-1 negatively correlated with 6 month BMI loss (r=-0.679, p=0.0001)[103].

Resistin, was upregulated in CC in gastric[103] and gastroesophageal cancer[104]. In gastric cancer, the concentration of resistin was 66.7 ng/ml in CC and 43.4 ng/ml WSC (p=<0.001), while in the gastroesophageal cohort, it was 11.74 ± 2.98 ng/ml in CC and 8.99 ± 3.21 ng/ml (P<0.001) in WSC. Resistin negatively correlated with 6 month BMI loss (r=-0.574, p<0.001) in gastric cancer[103]. In gastroesophageal cancer, resistin had an AUC of 0.71. The optimal cut-off point was 9.4 ng/mL and this had a sensitivity of 56% and specificity of 68%[104].

Leptin was identified as a biomarker of cachexia or BMI in n=3 studies. In oesophageal cancer, leptin was downregulated in CC compared to WSC (424 ng/L and 2122 ng/L respectively, p<0.001) and positively correlated with BMI (r= 0.815, p<0.0001 in women, r=0.687, p<0.0001 in men)[105]. Conversely, in gastric cancer, leptin was increased in CC compared to WSC (3405 ± 640 ng/L and 2623 ± 665 respectively, p=0.003) and negatively correlated with 6 month BMI loss (r=-0.438, p<0.001)[103]. In another study of oesophageal cancer, BMI weakly positively correlated with BMI (r=0.34)[106].

Adiponectin was upregulated in CC compared to WSC in gastric cancer (36.5 ± 15.0 μg/mL and 27.8 ± 11.9 respectively, 0.045) and negatively correlated with 6 month BMI loss (r=-0.283, p<0.028)[103]. It also weakly negatively correlated with BMI in oesophageal cancer (r=-0.26)[106] and was inversely associated with body weight in rectal neoplasia pre-surgery (r^2=0.11, p=0.045 in men, r^2=0.239, p=0.025 in women). Interestingly, this association disappeared in women post-surgery but remained in men[107]. However, prior to surgery, adiponectin was higher in women who lost weight compared to those who did not (p<0.05), while this was not the case for men[107].

Midkine had an AUC of 0.615 (p=0.045) for the identification of CC in gastroesophageal cancer patients. The cut-off value >2280 pg/mL had a sensitivity of 42% and a specificity of 85%. The average concentration of the hormone were 1375 pg/ml in WSC and 1975 pg/ml in CC (p=0.053)[108].

While not associated with cachexia per se, fibroblast growth factor 21 (FGF21) was significantly decreased in patients with low fat mass index (FMI), low fat mass percentage (FM%), and low subcutaneous adipose tissue index (SATI) (p=0.023,p=0.026 and p=0.007) and positively correlated with all three, FMI (r = 0.249, *P* = 0.044), FM% (r = 0.285, *P* = 0.02), and SATI (r = 0.346, *P* = 0.004). It did not correlate with any skeletal muscle related measures[109].

Table 4: Hormones identified as biomarkers in cachexia

	Hormones
Adiponectin (*n*=3)	• Adipokines and ghrelin in gastric cancer cachexia • Adipocytokines and squamous cell carcinoma of the esophagus • Leptin and adiponectin dynamics at patients with rectal neoplasm - Gender differences (women only)
Ghrelin (*n*=1)	• Adipokines and ghrelin in gastric cancer cachexia
Insulin (*n*=1)	• Adipokines and ghrelin in gastric cancer cachexia
IGF-1 (*n*=1)	• Adipokines and ghrelin in gastric cancer cachexia
Resistin (*n*=1)	• Adipokines and ghrelin in gastric cancer cachexia • Serum levels of resistin, adiponectin, and apelin in gastroesophageal cancer patients
Leptin (*n*=1)	• Circulating leptin and inflammatory response in esophageal cancer, esophageal cancer-related cachexia-anorexia syndrome (CAS) and non-malignant CAS of the alimentary tract • Adipocytokines and squamous cell carcinoma of the esophagus • Adipokines and ghrelin in gastric cancer cachexia
Midkine (*n*=1)	• Impact of weight loss on circulating IL-1, IL-6, IL-8, TNF-α, VEGF-A, VEGF-C and midkine in gastroesophageal cancer patients

FGF21 (*n*=1)	• Association between Plasma FGF21 Levels and Body Composition in Patients with Gastric Cancer

Lipid profile related markers:

In the included studies, *n*=3 lipid profile related biomarkers were identified as potential biomarkers of cachexia, High Density Lipoprotein (HDL) cholesterol, Low Density Lipoprotein (LDL) cholesterol and triglycerides (

Table 5).

Lima et al.,[110] and de Castro et al.,[111] found that HDL was significantly different (p=0.012 and p=0.0075 respectively) between weight stable cancer patients (WSC) compared to cancer patients with cachexia (CC). Lima et al., found that the mean HDL concentration in WSC was 39.45 ± 2.66 mg/dL compared to 31.81 ± 1.672 mg/dL in CC. Castro et al., found the mean HDL concentration was 44.32 ± 3.00 mg/dL in WSC and 35.75 ± 1.63 mg/dL in CC.

De Castrol et al., found that LDL was significantly different between WSC and CC (p=0.0443). The concentration of LDL was 114.15 mg/ml in WSC and 81 mg/ml in CC[111].

Lima et al., found that triglycerides were significantly different (p=0.031) between WSC and CC (126 mg/dL and 88mg/dL respectively)[110].

Table 5: Lipid profile related markers identified in cachexia

Lipid profile related markers

HDL cholesterol (*n*=2)	• Tumour-derived transforming growth factor-β signalling contributes to fibrosis in patients with cancer cachexia • Myokines in treatment-naive patients with cancer-associated cachexia
LDL cholesterol (*n*=1)	• Myokines in treatment-naive patients with cancer-associated cachexia
Triglycerides (*n*=1)	• Tumour-derived transforming growth factor-β signalling contributes to fibrosis in patients with cancer cachexia

Tumour markers and other proteins:

In the included studies, the tumour markers, immunosuppressive acidic protein and carcinoembryonic antigen, were identified along with insulin growth factor binding protein 3 (IGFBP-3), tissue inhibitor of metalloproteinases-1 (TIMP-1) and fatty acid binding protein 3 (FABP3) (Table 6).

Immunosuppressive acidic and carcinoembryonic antigen were positively, albeit weakly, correlated with weight loss (r=0.196, p=0.05 and r=0.281, p=0.0048). However, this was not corrected for other variables, such as tumour size which correlated with both weight loss and immunosuppressive acidic protein, and so should be interpreted with caution.

IGFBP3 was significantly upregulated in the serum of CC compared to WSC ($p<0.05$) (~700pg/ml and ~1100pg/ml respectively, exact values not given as data is graphed)[63].

Plasma TIMP1 positively correlated with weight loss in a colorectal cancer cohort ($r=0.221, p=0.046$). Additionally, high levels of TIMP1 combined with weight loss resulted in shorter survival compared to those with weight loss and low TIMP1[112].

Plasma FABP3 was significantly higher in CC compared to WSC ($p=0.0301$) in a cohort of patients with gastric or colorectal cancer. This reflected higher tumour levels of the protein in CC compared to WSC ($P=0.0182$)[113].

Table 6: Tumour and other markers identified as biomarkers in cachexia

Tumour markers and other proteins	
Immunosuppressive acidic protein (n=1)	• Serum immunosuppressive acidic protein reflects systemic deterioration of colorectal cancer patient condition
Carcinoembryonic antigen (n=1)	• Serum immunosuppressive acidic protein reflects systemic deterioration of colorectal cancer patient condition
IGFBP-3 (n=1)	• IGFBP-3 promotes cachexia-associated lipid loss by suppressing insulin-like growth factor/insulin signaling
TIMP-1 (n=1)	• A novel tissue inhibitor of metalloproteinases-1/liver/cachexia score predicts prognosis of gastrointestinal cancer patients
FABP3 (n=1)	• Myokines in treatment-naive patients with cancer-associated cachexia

Sarcopenia

There were various definitions used to define sarcopenia in the included papers. The Martin et al.,[47] definition was used by *n*=5 studies, while *n*=1 study used very similar cut-off points to this definition. Study specific cut-off points were used by *n*=9 studies. Strategies included stratifying patients into quartiles based on muscle mass, while other studies used a median cut-off or standard deviation. Of the included studies, *n*=8 used EWGSOP/AWGSOP/AWGS criteria which included low muscle mass and low muscle strength (handgrip) or reduced functionality to define sarcopenia, however the studies using theses definitions did not use the same SMI cut-off values. Also included were *n*=3 studies which investigated more than 1 definition (Supplementary table 2). Prevalence of sarcopenia ranged from 14.9%-85.7% with a median of 42.4%.

Biomarkers:

In the included studies, *n*=31 potential biomarkers of sarcopenia in cancer were identified. These were classified by their most relevant function into inflammatory biomarkers, metabolites and nutrition related biomarkers, hormones and tumour other markers which will be discussed below. Tables are organised in descending order of biomarkers most commonly identified in the literature.

Inflammatory markers:

From the included studies, *n*=20 inflammatory markers were identified including acute phase proteins (CRP/HS-CRP, albumin), cytokines (IL-8, TNFα and interferon-induced protein 10 (ip-10)), cells

(Neutrophils, monocytes, platelets and leucocytes) and various scores and ratios (mGPS, NLR, Lymphocyte-monocyte ratio etc) (Table 7). CRP and albumin were the most common markers identified (*n*=6 and *n*=15, respectively), followed by mGPS and NLR (*n*=4 for each).

CRP/HS-CRP was associated with sarcopenia in *n*=6 studies. CRP >5mg/l was considered elevated in *n*=2 studies of patients with colorectal cancer. Elevated CRP was present in 73.8% of patients with sarcopenia (S) compared to 51.1% of patients without sarcopenia (WS) (p=0.029) in one study[114] and in 40% of S compared to 15% of WS (p<0.0001) in another[115]. CRP was weakly correlated with skeletal muscle in one study (r=-0.21, p=0.005)[116], and with skeletal muscle index (SMI) (r=− 0.360, p=0.010) and handgrip-strength in another (r=− 0.476, p< 0.001)[117]. CRP increased with the severity of sarcopenia in oesophageal cancer[118]. HS-CRP was also increased in S compared to WS (3.91 mg/dl and 0.75 mg/dl, p<0.001) prior to chemotherapy. Patients who developed sarcopenia post-chemotherapy had significantly increased HS-CRP (1.55 mg/dl) compared to pre-chemotherapy (0.51 mg/dl) (p=0.001) while those who did not develop sarcopenia remained unchanged[119].

Low albumin concentration was associated with S in *n*=15 studies. Levels ranged from 26.4 g/l to 41 g/l in S and 30.8 g/l to 44 g/l in WS. In one cohort of colorectal cancer, albumin correlated with SMI (r=0.31, p<0.001) and visceral fat (r=0.18, p=0.02)[116]. In a cohort of patients with oesophageal cancer, albumin was significantly different between S and WS (40 g/l and 38 g/l, p=0.022) only when using the modified European Working Group on Sarcopenia in Older People (mEWGSOP) definition but not when using other definitions of sarcopenia such as EWGSOP and Asian Working Group for Sarcopenia (AWGS)[120]. In patients with gastric cancer, albumin had an AUC of 0.607 (p=0.013)[121].

Elevated mGPS (CRP >10 mg/l + albumin <35 g/l) was associated with S in *n*=4 studies. CRP/albumin ratio was also significantly higher in patients with S in a cohort of patients with oesophageal cancer, while in another oesophageal cancer cohort, the geriatric nutrition index (GRNI) (albumin x body weight) was significantly associated with SMI (p<0.001)[122].

Also identified as potential biomarkers of S were different cell types and various scores/ratios. Neutrophils (r=0.097, p=0.022), monocytes (r=-0.126, p=0.003) and leucocytes (r=0.135, p=0.001) correlated with SMI, albeit weakly, in n=1 study of patients with colon cancer[123]. Lymphocytes also correlated with SMI in the same study (r=0.142, p=0.001)[123], and were decreased in S compared to WS in patients with gastric cancer (1160.61 ± 990.0 count/µl and 1520.90 ± 1030.1 count/µl)[121]. Platelets were significantly correlated with SMI (r=-0.101, p=0.017) in colon cancer[123] and were significantly decreased in S compared to WS in oesophageal cancer (267 ± 77 count/µl and 237 ± 65 count/µl, *p*=0.016). A high Neutrophil to lymphocyte ratio was identified as a marker of S in *n*=4 studies. A high

(>40[124], and >45.35[122]) Prognostic Nutritional Index (PNI) (albumin + total number of peripheral blood lymphocytes) was significantly associated with sarcopenia in esophageal[122] and gastric cancer[124].

IL-8 was significantly increased in S compared to WS (146.02 ± 311.96 pg/ml and 47.24 ± 66.3 pg/ml, p=0.009) in pre-treatment patients with colorectal or gastric cancer. It also correlated with pre- and post-treatment SMI (r=− 0.510, p< 0.001 and r=− 0.431, p=0.001 respectively) and pre- and post-treatment handgrip-strength (r=− 0.397, p< 0.001 and r=− 0.418, p=0.001 respectively)[119]. TNFα was negatively associated with fat free mass (r=-0.394, p=0.008) and food intake (r=-0.389, p=0.010) in a cohort of patients with oesophageal, gastric or colorectal cancer[125].

Table 7: Inflammatory markers identified in sarcopenia

Inflammatory markers

Albumin (*n*=15)	• Association of low skeletal muscle index with increased systematic inflammatory responses and interferon γ-induced protein 10 levels in patients with colon cancer • Development and validation of risk prediction model for sarcopenia in patients with colorectal cancer • Sarcopenia and Myosteatosis Are Associated with Neutrophil to Lymphocyte Ratio but Not Glasgow Prognostic Score in Colorectal Cancer Patients • The relationships between body composition and the systemic inflammatory response in patients with primary operable colorectal cancer • Factors Associated with Sarcopenia in Patients with Colorectal Cancer • Close Relationship Between Immunological/Inflammatory Markers and Myopenia and Myosteatosis in Patients With Colorectal Cancer: A Propensity Score Matching Analysis • The Optimal Definition of Sarcopenia for Predicting Postoperative Pneumonia after Esophagectomy in Patients with Esophageal Cancer • Preoperative low muscle mass and malnutrition affect the clinical prognosis of locally advanced gastric cancer patients undergoing radical surgery • Adverse effects of preoperative sarcopenia on postoperative complications of patients with gastric cancer • Clinical significance of sarcopenia in patients undergoing treatment for gastric cancer • Sarcopenia: a new predictor of postoperative complications for elderly gastric cancer patients who underwent radical gastrectomy • Associations between skeletal muscle mass index, nutritional and functional status of patients with oesophago-gastric cancer • Low Muscularity and Myosteatosis Is Related to the Host Systemic Inflammatory Response in Patients Undergoing Surgery for Colorectal Cancer. • Circulating miR-203 derived from metastatic tissues promotes myopenia in colorectal cancer patients • Development and validation of nomograms for the prediction of low muscle mass and radiodensity in gastric cancer patients
CRP/HS-CRP* (*n*=6)	• The relationships between body composition and the systemic inflammatory response in patients with primary operable colorectal cancer • Close Relationship Between Immunological/Inflammatory Markers and Myopenia and Myosteatosis in Patients With Colorectal Cancer: A Propensity Score Matching Analysis • The relationship between sarcopenia detected in newly diagnosed colorectal cancer patients and FGF21, irisin and CRP levels • Host phenotype is associated with reduced survival independent of tumour biology in patients with colorectal liver metastases

	• The evaluation of the association between preoperative sarcopenia and postoperative pneumonia and factors for preoperative sarcopenia in patients undergoing thoracoscopic-laparoscopic esophagectomy for esophageal cancer • The role of inflammation in adjuvant chemotherapy-induced sarcopenia (Izmir Oncology Group (IZOG) study*
mGPS (*n*=4)	• The relationship between computed tomography-derived body composition, systemic inflammatory response, and survival in patients undergoing surgery for colorectal cancer • The relationships between body composition and the systemic inflammatory response in patients with primary operable colorectal cancer • The relationship between tumour stage, systemic inflammation, body composition and survival in patients with colorectal cancer • Prognostic significance of sarcopenia and systemic inflammatory response in patients with esophageal cancer
NLR (*n*=4)	• Association of low skeletal muscle index with increased systematic inflammatory responses and interferon Y-induced protein 10 levels in patients with colon cancer • Development and validation of risk prediction model for sarcopenia in patients with colorectal cancer • Sarcopenia and Myosteatosis Are Associated with Neutrophil to Lymphocyte Ratio but Not Glasgow Prognostic Score in Colorectal Cancer Patients • Prognostic significance of the l3 skeletal muscle index and advanced lung cancer inflammation index in elderly patients with esophageal cancer
Lymphocyte-monocyte ratio (*n*=2)	• Close Relationship Between Immunological/Inflammatory Markers and Myopenia and Myosteatosis in Patients With Colorectal Cancer: A Propensity Score Matching Analysis • Prognostic value of sarcopenia and systemic inflammation markers in patients undergoing definitive radiotherapy for esophageal cancer
Lymphocytes (*n*=2)	• Association of low skeletal muscle index with increased systematic inflammatory responses and interferon γ-induced protein 10 levels in patients with colon cancer • Clinical significance of sarcopenia in patients undergoing treatment for gastric cancer
Platelets (*n*=2)	• Association of low skeletal muscle index with increased systematic inflammatory responses and interferon γ-induced protein 10 levels in patients with colon cancer • Prognostic significance of sarcopenia and systemic inflammatory response in patients with esophageal cancer
Prognostic nutritional index (PNI): serum albumin concentration and the total number of peripheral blood lymphocytes (*n*=2)	• Prognostic significance of the l3 skeletal muscle index and advanced lung cancer inflammation index in elderly patients with esophageal cancer • The combination of body composition conditions and systemic inflammatory markers has prognostic value for patients with gastric cancer treated with adjuvant chemoradiotherapy
Pre-albumin (*n*=1)	• Combined test of third lumbar skeletal muscle index and prognostic nutrition index improve prognosis prediction power in resected colorectal cancer liver metastasis
CRP/albumin ratio (*n*=1)	• Prognostic significance of sarcopenia and systemic inflammatory response in patients with esophageal cancer
Neutrophils (*n*=1)	• Association of low skeletal muscle index with increased systematic inflammatory responses and interferon γ-induced protein 10 levels in patients with colon cancer
Monocytes (*n*=1)	• Association of low skeletal muscle index with increased systematic inflammatory responses and interferon γ-induced protein 10 levels in patients with colon cancer
Leukocyte (*n*=1)	• Association of low skeletal muscle index with increased systematic inflammatory responses and interferon Y-induced protein 10 levels in patients with colon cancer
Neutrophil platelet score (*n*=1)	• Close Relationship Between Immunological/Inflammatory Markers and Myopenia and Myosteatosis in Patients With Colorectal Cancer: A Propensity Score Matching Analysis

Systemic immune-inflammation index (Platelet count (× 109/L) × neutrophil count (× 109/L) / lymphocyte count) (*n*=1)	• Close Relationship Between Immunological/Inflammatory Markers and Myopenia and Myosteatosis in Patients With Colorectal Cancer: A Propensity Score Matching Analysis
IL-8 (*n*=1)	• The role of inflammation in adjuvant chemotherapy-induced sarcopenia (Izmir Oncology Group (IZOG) study
TNFα (*n*=1)	• Inflammatory cytokines, appetite-regulating hormones, and energy metabolism in patients with gastrointestinal cancer
Interferon-induced protein 10 (ip-10) (*n*=1)	• Association of low skeletal muscle index with increased systematic inflammatory responses and interferon Y-induced protein 10 levels in patients with colon cancer
GNRI: albumin * body weight (*n*=1)	• Prognostic significance of the l3 skeletal muscle index and advanced lung cancer inflammation index in elderly patients with esophageal cancer
ALBI score: log10bilirubin [mol/L]×0.66) + (Albumin [g/L]×0.0852) (*n*=1)	• Association of Albumin-Bilirubin Grade and Myosteatosis with its Prognostic Significance for Patients with Colorectal Cancer

Metabolites and nutrition related markers:

In the included studies, *n*=5 metabolite and nutritional related markers were identified (Table 8), however, n=3 of these (total protein, calcium and uric acid) were identified in only one cohort of patients with colorectal cancer[126]. In that study, total protein, calcium and uric acid were decreased in S compared to WS and were significantly associated in an univariate analysis (p<0.001, p<0.001 and p=0.013 respectively)[126].

A low creatine x albumin score was associated with a higher percentage of patients with S (p=0.0003), and a lower SMI in men but not in women (p=0.003)[127].

Haemoglobin was decreased in S compared to WS in n=8 studies. Concentrations ranged from 9.8 g/dl to 12.7 g/dl in S and 10.4 g/dl to 13 g/dl in WS. In a study of patients with colorectal cancer, haemoglobin was significantly decreased in S compared to WS in the training cohort (11.8 g/dl and 12.7 g/dl, p<0.001) but not in the validation cohort (12.1 g/dl and 12.7 g/dl, p=0.199)[128]. In patients with oesophageal cancer, haemoglobin was associated with sarcopenia in univariate analysis (p=0.021) but not in multivariate analysis.

Table 8: Metabolites and nutrition related markers in sarcopenia

Metabolites and nutrition related markers

Haemoglobin (*n*=8)	• Development and validation of risk prediction model for sarcopenia in patients with colorectal cancer • The relationships between body composition and the systemic • Establishment and validation of novel nomograms to predict muscle quality in colorectal cancer patients inflammatory response in patients with primary operable colorectal cancer • The evaluation of the association between preoperative sarcopenia and postoperative pneumonia and factors for preoperative sarcopenia in patients undergoing thoracoscopic-laparoscopic esophagectomy for esophageal cancer • Clinical significance of sarcopenia in patients undergoing treatment for gastric cancer • Sarcopenia: a new predictor of postoperative complications for elderly gastric cancer patients who underwent radical gastrectomy

	• Circulating miR-203 derived from metastatic tissues promotes myopenia in colorectal cancer patients • Development and validation of nomograms for the prediction of low muscle mass and radiodensity in gastric cancer patients
Total protein (*n*=1)	• Development and validation of risk prediction model for sarcopenia in patients with colorectal cancer
Calcium (*n*=1)	• Development and validation of risk prediction model for sarcopenia in patients with colorectal cancer
Uric acid (*n*=1)	• Development and validation of risk prediction model for sarcopenia in patients with colorectal cancer
Creatinine x Albumin (*n*=1)	• Actual Sarcopenia Reflects Poor Prognosis in Patients with Esophageal Cancer

<u>Tumour markers and other proteins:</u>

In the included studies, *n*=4 tumour markers and proteins were identified as biomarkers of sarcopenia (Table 9). Each biomarker was identified in one study each.

In a cohort of patients with colorectal cancer, patients were stratified into 4 quartiles based on muscle mass. Carcinoembryonic antigen, was increased in patients in Q4 (lowest muscle mass) compared to Q1 (highest muscle mass) (3.4 U/ml and 2.0 U/ml). However, it is interesting to note that the range varied largely (0.2–527.3 U/ml) and the concentration did not increase linearly from Q1 to Q4 (Q1 (2.0 U/ml), Q2 (1.6 U/ml), Q3 (2.2 U/ml), Q4 (3.4 U/ml)[129].

Creatine kinase was significantly decreased in S compared to WS in patients with colorectal cancer (49 U/L and 67 U/L, p=0.005)[126].

Serum MiR-203 was increased in patients with a low psoas muscle index (PMI) (p=0.014) and also negatively correlated with PMI (r=-0.25, p=0.001). Pre-surgery serum MiR-203 predicted post-surgery muscle wasting in both univariate and multivariate analysis (p=0.0001 and p=0.002 respectively)[130].

Low serum cholinesterase (< 228 U/L) was associated with S (p<0.001) and SMI (p<0.001) in colorectal cancer[131].

Table 9: Tumour and other markers identified in sarcopenia

Tumour markers and other proteins

Carcinoembryonic antigen (*n*=1)	• Sarcopenia is a Negative Prognostic Factor After Curative Resection of Colorectal Cancer
Creatine Kinase *n*=1)	• Development and validation of risk prediction model for sarcopenia in patients with colorectal cancer
MiR-203 (*n*=1)	• Circulating miR-203 derived from metastatic tissues promotes myopenia in colorectal cancer patients
Cholinesterase (*n*=1)	• The influence of serum cholinesterase levels and sarcopenia on postoperative infectious complications in colorectal cancer surgery

Hormones:

In the included studies, *n*=2 hormones were associated with S in the same study of patients with colorectal cancer, irisin and FGF21 (Table 10). Irisin was decreased in S compared to WS (76.1 pg/ml and 256.3 pg/ml, p<0.001) and correlated with SMI (r=0.564,p<0.001) and handgrip-strength (r=0.290, p=0.041). FGF21 was also decreased in S compared to WS (55.8 pg/ml and 175.3 pg/ml, p=0.007) and correlated with SMI (r=0.282,p=0.048) and handgrip-strength (r=0.342, p=0.015).

Table 10: Hormones identified in Sarcopenia

Hormones	
Irisin (*n*=1)	• The relationship between sarcopenia detected in newly diagnosed colorectal cancer patients and FGF21, irisin and CRP levels
FGF21 (*n*=1)	• The relationship between sarcopenia detected in newly diagnosed colorectal cancer patients and FGF21, irisin and CRP levels

Markers identified as a marker of both cachexia and sarcopenia:

In total, *n*=5 markers were identified as biomarkers of both cachexia and sarcopenia (Figure 2). The majority of these are inflammatory based markers and scores such as the mGPS, CRP, TNFα, albumin and neutrophil-lymphocyte count.

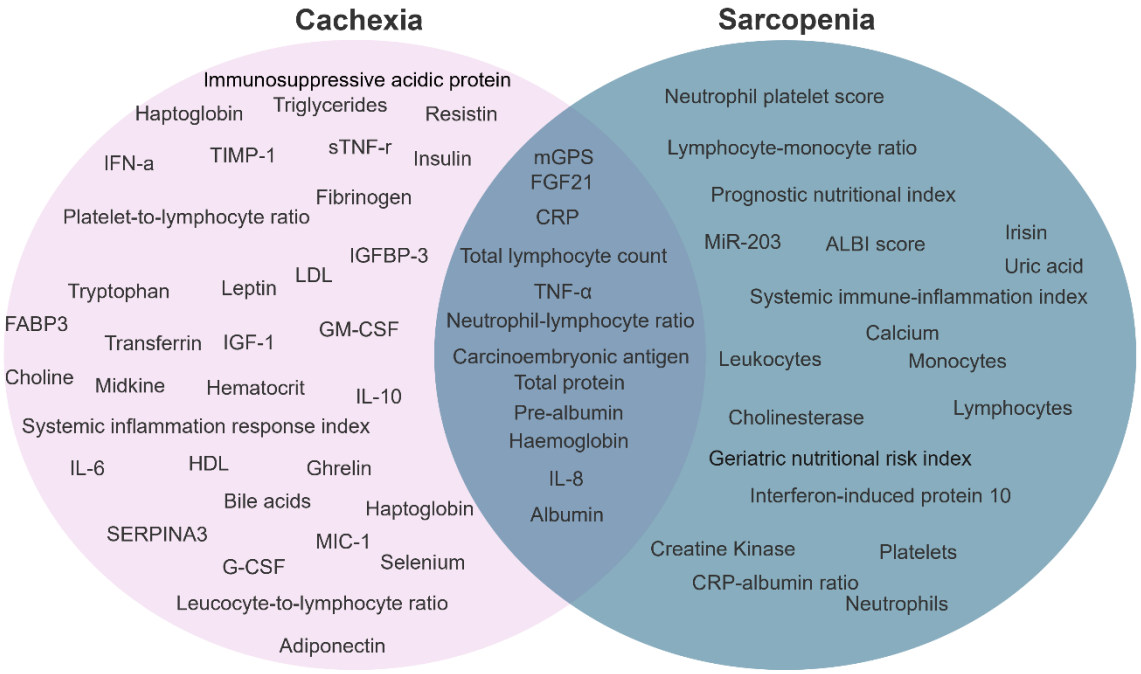

Figure 2: Venn diagram of markers identified as biomarkers of cachexia and/or sarcopenia in the screened papers.

Discussion

This rapid review focused on circulatory biomarkers identified in serum, plasma and whole blood as they are the most adaptable to clinical use. In total, *n*=73 biomarkers were identified and grouped based on the most relevant function, with inflammatory (*n*=38) and metabolites and nutrition related markers (*n*=14) the most commonly identified. Unsurprisingly, markers that are measured routinely in the hospital setting, including CRP, albumin and haemoglobin, were some of the most commonly identified markers of cachexia and sarcopenia. CRP is a broad spectrum marker of inflammation[70] and so it is unsurprising that it was upregulated in patients with cachexia and sarcopenia. Indeed some of the current definitions and screening tools for cachexia use elevated CRP (generally >10g/L) as a criterion for diagnosis, such as the Evans et al.,[34] definition and GLIM screening tool[35]. Additionally, elevated CRP has been associated with reduced muscle mass and strength in a meta-analysis of *n*=76,899 community dwelling participants[132]. While CRP certainly has its place in the diagnosis of cachexia and sarcopenia, it is not an ideal biomarker due to its lack of specificity[133]. It is also lacks consistency as a biomarker of cachexia and sarcopenia. In the studies investigated here, *n*=13 studies identified CRP/HS-CRP as a biomarker of cachexia and *n*=6 studies identified CRP/HS-CRP as a biomarker of sarcopenia. However, CRP was also not associated with cachexia in *n*=3 studies[134-136] or sarcopenia in *n*=7 studies[11, 120, 123, 137-140]. This potentially highlights CRP is also a better biomarker of cachexia compared to sarcopenia. Albumin was identified as a biomarker of cachexia *n*=11 studies and sarcopenia in *n*=15 studies. Albumin is the most abundant protein in blood[141] and functions as a transport protein[142] and regulator of vascular osmotic pressure[143] with anti-inflammatory[144, 145] and anti-oxidative[146] properties. It is a negative acute phase protein and is therefore reduced in response to inflammation[147]. It has previously been associated with sarcopenia in people with[148] and without cancer[149-151] and cachexia[152]. Interestingly, in a cohort of healthy young and old participants, albumin was associated with muscle mass in young but not in older subjects after adjusting for CRP, age, body mass and lifestyle factors[153]. However, like CRP, albumin is a non-specific marker of inflammation[154]. Additionally, the half-life of albumin is ~21 days[155] and so it may not accurately reflect the current status of the patient, making it a less than ideal biomarker for monitoring cachexia and sarcopenia. Albumin was not associated with cachexia in *n*=4[98, 111, 134, 135] of the included studies and with sarcopenia in *n*=3[122, 139, 156] studies. Pre-albumin may be a more suitable biomarker of cachexia and sarcopenia due to its shorter half-life (~2 days)[157]. It was identified as a biomarker of cachexia in *n*=3 studies and in sarcopenia in *n*=1 study. Interestingly, in one included study, prealbumin correlated with weight loss and was decreased in malnutrition in patients with oesophageal cancer while albumin did not[98]. Given the association of CRP and albumin with cachexia and sarcopenia, a number of screening tools have been developed that include one or both of these proteins. The modified Glasgow

prognostic score (mGPS) is an excellent example of this and combines low albumin (<35g/L) and high CRP (>10 mg/L) to determine cachexia risk. In 2014 Douglas and McMillan[158] proposed a mGPS score of 0 for those with normal CRP ± normal albumin, a score of 1 for those with high CRP but normal albumin and a score of 2 for those with high CRP and low albumin. They also proposed that those with a mGPS score of 1 be considered “pre cachexia” while those with a score of 2 be considered "refractory cachexia”[158]. Da Silva et al., investigated the clinical utility of this score in patients during palliative care (*n*=1166) and found it significantly associated with survival (77 days in “no cachexia patients” and 17 days in the “refractory cachexia” patients). However, while there was significantly more weight loss in the patients with “refractory cachexia”, ~60% of patients with “no cachexia” experienced weight loss >5% at 6 months[159]. In a study by Dolan et al., mGPS was not associated with low SMI but was associated with low skeletal muscle density, time to up and go and handgrip strength[160]. In the included studies, mGPS was considered a biomarker of cachexia in *n*=2 studies and sarcopenia in *n*=4 studies, suggesting it may be more representative of sarcopenia compared to cachexia. The mGPS was not associated with sarcopenia in one included study of patients with colorectal cancer, despite sarcopenia being associated with NLR and low albumin[137]. Haemoglobin is another protein commonly measured in the clinic. It was identified as a biomarker of cachexia in *n*=7 included studies and sarcopenia in *n*=8 studies. Haemoglobin is a biomarker of malnutrition, specifically anaemia and is decreased in sarcopenia in people with[161] and without[162-164] cancer and during cachexia[161, 165]. Reduced haemoglobin in cancer patients can be caused by reduced iron availability due to bleeding[166] anorexia or malabsorption[166, 167], chemotherapy induced erythropoiesis disturbance[168] or inflammation[169]. Haemoglobin was not associated with cachexia in *n*=2[135, 136] included studies and with sarcopenia in *n*=1[170] study.

The cytokines, IL-6, IL-8 and TNFα were particularly prevalent in cachexia. IL-6 is a well known biomarker and inducer of cancer cachexia and has been implicated in both muscle and adipose tissue wasting. Pre-clinical models have identified several IL-6 mediated mechanisms that promote muscle wasting such as suppression of protein synthesis through mechanistic target of rapamycin (mTOR) [171], promotion of the ubiquitin proteasome pathway[172-174], autophagy[172, 173, 175] and apoptosis[176]. IL-6 may also promote adipose tissue loss through lipolysis[96, 177], and white adipose tissue browning[96, 178]. The promotion of white adipose tissue browning by IL-6 increases energy expenditure through thermogenesis[175]. IL-6 may also promote increased energy expenditure through the induction of the acute phase response as shown in patients with pancreatic cancer[179]. Despite being identified as a biomarker of cachexia in several cancer types[180] including lung[65, 152] head and neck[181] and the *n*=8 gastrointestinal cancer studies included here, it has not been consistent[182, 183]. In this review, IL-6 was not associated with cachexia in *n*=3 studies or not associated with sarcopenia in *n*=3 studies.

Additionally, IL-6 levels can be affected by age[184], obesity[185, 186] and smoking[187] which may hinder its utility as a biomarker of cachexia. IL-8 is a pro-inflammatory cytokine[188] produced from a variety of cells, including tumour[189] and immune cells[190, 191], that promotes the recruitment of neutrophils to sites of inflammation[192]. It has several tumour promoting roles such as the stimulation of angiogenesis through the expression of VEGF[193] and increased tumour cell proliferation and metastasis[194-196]. Increased IL-8 is considered a negative prognostic marker in cancer, indicating reduced survival and increased reoccurrence[196-198]. IL-8 has also been identified as a biomarker of cachexia[180] but its association with sarcopenia is less certain[199, 200]. This is reflected in the studies included in our review as only *n*=1 study identified IL-8 as a biomarker of sarcopenia[119]. However, IL-8 was associated with sarcopenia in patients with pancreatic cancer and negatively correlated with total psoas muscle area (r=-0.2544)(*n*=93)[200]. Callaway et al., showed that IL-8 induced muscle atrophy via extracellular signal-regulated kinase 1/2 (ERK1/2), signal transducer and activator of transcription (STAT), and Suppressor of mother against decapentaplegic (SMAD) signaling[201] while exosomal IL-8 from lung and colon cancer cells induced adipocyte lipolysis via NF-κB[202]. IL-8 was not associated with cachexia in *n*=2[134, 203] studies included in this review and with sarcopenia in *n*=1[123] study. As outlined in the introduction, TNFα is one of the original biomarkers of cancer cachexia that has failed to reach the clinic for a variety of reasons, one of these being its lack of consistency. Indeed in this review, *n*=10 studies investigated TNFα in cachexia and of these *n*=6[108, 111, 203-206] found no association. In sarcopenia, TNFα was investigated in *n*=3 studies and was not associated with the condition in *n*=2[119, 123].

Interestingly, more hormones were identified as biomarkers of cachexia than sarcopenia in this review. Leptin and adiponectin were the most commonly identified hormone biomarkers, (identified in *n*=3 studies each), which probably represents the loss of adipose tissue, where both are derived from, which is characteristic of both conditions. Leptin, the satiety hormone, is produced in adipocytes and concentrations reflect total fat mass[207]. Leptin promotes satiety in the hypothalamus by activating neurons containing proopiomelanocortin-containing (POMC) and inactivating neurons containing gouti-related protein/neuropeptide Y-containing (AgRP/NPY)[208]. Decreases in leptin due to reduced body stores typically promote hunger and a decrease in energy expenditure[208] while high levels increase energy expenditure[209]. It has also plays a role in the immune system, as reviewed by La Cava and Matarese[210], and can promote the induction of pro-inflammatory cytokines such as IL-6[210]. In the included studies, leptin was decreased in patients with cachexia and oesophageal cancer and continued to be associated with cachexia after adjusting for BMI[105]. In patients with gastric cancer, leptin was increased in cachexia compared to patients without cachexia and negatively correlated with 6 month BMI change (%), meaning those who lost the most weight had high amounts of leptin[103]. A study of breast and colon cancer patients found that leptin corelated with weight loss in women with

cachexia but not men[211]. While a systematic review determined that there was no significant difference between patients with cachexia and patients without in several cancer types[180]. The variability between studies and the direct effects of BMI make leptin a challenging biomarker for cachexia, particularly with a high prevalence of obesity. Adiponectin is also secreted by adipocytes but unlike leptin, levels of the hormone are negatively associated with fat mass and increase with weight loss[212]. Adiponectin is protective against insulin resistance, promoting glucose uptake by skeletal muscle via GLUT4[213] and glucose utilisation[214] and sensitivity[215] in adipocytes. It also plays a role in lipid metabolism by promoting the utilisation of fatty acids via β-oxidation in muscle[214, 216], promoting storage of triglycerides in adipocytes[215] and reducing the triglyceride content of muscle and liver[212, 216]. Adiponectin levels were increased in patients with cachexia and/or negatively correlated with BMI in the studies included here, however many studies report no difference in response to cachexia[182, 217, 218] and a systematic review by Paval et al., determined that it was not a marker of cachexia in multiple cancer types[180]. FGF21 was decreased in response to sarcopenia in a cohort of colorectal cancer patients and correlated with handgrip-strength and SMI, mimicking a study in *n*=125 community dwelling older people without cancer[219]. FGF21, is a mitokine, a marker of mitochondrial stress and is increased during ageing[220] and protein restriction[221]. However, a recent meta-analysis of FGF21 in *n*=635 people with sarcopenia found that FGF21 was not associated with the disorder[222]. More research is needed to determine if FGF21 is a biomarker of sarcopenia in cancer.

In conclusion, this rapid review summarised circulating biomarkers of cachexia and sarcopenia in patients with gastric, colorectal and oesophageal cancer. Potential biomarkers were predominantly markers of inflammation, while the most frequently identified biomarkers were measured routinely in the clinic. Many of the biomarkers were not consistently associated with cachexia or sarcopenia despite the small number of cancer types included, highlighting the reproducibility crisis facing biomarker discovery. As many of the identified biomarkers are not specific to cachexia or sarcopenia, it is quite possible a multi-marker approach is needed to create a unique signature of these disorders. In this thesis we will explore a potential solution to this problem by using the proteome of high density lipoprotein (HDL), as a biomarker. HDL has a diverse proteome with ~285 identified proteins involved in inflammation, oxidative stress and immunity[223]. The literature shows that the HDL proteome can be modulated by inflammatory and metabolic conditions[224, 225 226], becoming enriched with acute phase proteins such as serum amyloid A (SAA) and ceruloplasmin and depleted of others such as its characteristic protein, Apolipoprotein A1 (ApoA1)[227]. It is these changes that we hope to harness to create a unique, multi-protein, signature for the identification of cancer cachexia and sarcopenia.

In this rapid review, many of the biomarkers were identified in only *n*=1 study and so may represent biomarkers that need further exploration, such as MIC-1/GDF-15 which is an emerging biomarker of

cachexia[228]. It is important to take into consideration the broad number of definitions used to define cachexia and sarcopenia in this rapid review. We included papers that used any definition as to not exclude potential biomarkers and this may have had an effect on the biomarkers identified. This is particularly true given the diverse populations used within the included studies, as the definition of sarcopenia should be tailored to the ethnicity of the cohort[229]. It should be noted that, due to the short-cuts taken as part of the rapid review process, it is possible key papers were not included and therefore some biomarkers may be underrepresented. Furthermore, colorectal cancer was over-represented in the included studies and this may affect the applicability of the identified biomarkers in other cancer types. More research is needed in this space to identify specific, reproducible and accessible biomarkers that can be translated into the clinic.

Supplementary Material

Supplementary Table 1: Included studies that identified biomarkers of cachexia

Reference	Journal	Location	n number	Cancer type	Age	Sex	Treatment status of cohort	Body composition measurements	Cachexia Definition	Sarcopenia definition/cut off for low SMI handgrip etc	Sample type	Biomarker	Biomarkers examined that had no difference between groups
Toiyama, Y et al., 2008[230]	Journal of surgical oncology	Japan	Cancer patients =101, healthy controls =80	Colorectal	~66 (37-86)	M &F	Pre-surgery and chemotherapy/radiation	Weight loss: self reported	Correlation, no cut-off given	N/A	Serum	immunosuppressive acidic, carcinoembryonic antigen	
Brewczyński A., 2017[92]	Nutrition and cancer	Not stated but authors are from Poland	No weight loss (n=63), weight loss≤ 10% (n=65), weight loss >10% (n=79), total (n=207)	Gastric	~57.8 (25-74)	M &F	Pre-surgery and chemotherapy/radiation	Weight loss: self reported	Weight loss >10% in 3 months	N/A	Serum	Total lymphocyte count, albumin, total protein, Haemoglobin	
Costa RGF et al., 2019[231]	Journal cachexia, sarcopenia, and muscle	Sao Paulo, Brazil	Cachexia (n=25), Weight stable (n=20), total (n=45)	Colon cancer	~64	M &F	Pre-surgery and chemotherapy/radiation	Height and weight measured at enrolment but weight loss not stated as self-reported or from charts	Evans et al.,[34]	N/A	Plasma	IL-6,IL-8,CRP,albumin,haemoglobin,	TNF-α
Thibaut MM et al., 2021[100]	Journal cachexia, sarcopenia, and muscle	Brussels, Belgium	Cachexia (n=43), Weight stable (n=51), total (n=94)	Colorectal cancer	~60's (25-95)	M &F	Pre-surgery and chemotherapy/radiation	Skeletal muscle CT image at third lumbar vertebra	Fearon et al.,[2]	N/A	Serum	Bile acids: glycoursodeoxycholic acid, TCDCA, total bile acids. IL-6, bilirubin	Alkaline phosphatase
Wang X, et al., 2023[63]	Chinese medical Journal	Beijing, China	Cachexia (n=14), Weight stable (n=31), total (n=45)	Colorectal cancer	~65	M &F	Not reported	Weight loss: self reported	weight loss >5% within the past six months with clinical phenotype or body mass index (BMI) <20 kg/m 2	N/A	Serum	IGFBP-3	

Lima JDCC, 2019[110]	Journal cachexia, sarcopenia, and muscle	Porto Alegre, Brazil	Cachexia (n=43), Weight stable (n= 31), total (n=74)	Colorectal cancer	~60s	M &F	Pre-surgery and chemotherapy/radiation	Weight loss: self reported, current weight, BMI	Evans et al.,[34]	N/A	Serum + plasma	CRP, haemoglobin, albumin, triglycerides, HDL, IL-8, IFN-a, G-CSF, GM?CSF	Total cholesterol, glucose, IL-12p40, IL-12p70, EGF, VEGF
Diakowska, D et al., 2010[134]	Cytokine	Not reported but authors are from Poland and ethics granted in Wroclaw	Cancer patients: Cachexia (n=84), Weight stable (n=51), Non-cancer controls: Cachexia (n=20), Weight stable (n=63)	Oesophageal	~60's	M &F	Pre-surgery and chemotherapy/radiation	Weight loss	>5% weight loss in 3 months	N/A	Serum	Leptin (females not males), IL-6, albumin, , TNF (only output that correlated with weight loss)	CRP, IL-1, IL-8, Total lymphocyte count, haemoglobin
Aydin, Y. et al., 2012[232]	Turkish Journal of Medical Sciences	Erzurum, rkeyT	Cachexia (n=32), Weight stable (n=11), Healthy control (n=43),total (n=86)	Oesophageal	~61.03	M &F	Pre-surgery and chemotherapy/radiation	Weight loss: self reported	Patients grouped as those who lost weight vs those who didn't but no value is given	N/A	Plasma	TNFα, IL-6	
Wu, J. et al., 2013[205]	Nutrition	Xin Hua, China	Cachexia (n=24), Weight stable (n=32), healthy controls (n=30), total (n=84)	Oesophageal	~60's	M	Pre-chemo/radiotherapy but not sure if pre-surgery	Weight: Scale, Fat mass, fat free mass, fat %	5% WL during the previous 3 mo or more than 10% WL during the previous 6 months	N/A	Serum	hs-CRP,IL-6,albumin,prealbumin	TNFα
Kerem, M. et al., 2008[103]	World Journal of gastroenterology	Ankara, Turkey	Cachexia (n=30), Weight stable (n=30), Healthy control (n=30), total (n=90)	Gastric	~50-60's	M &F	Pre-surgery and chemotherapy/radiation	Weight loss: self reported	> 10% reduction in BMI within 6 months prior to admission,	N/A	Serum	Haemoglobin, ghrelin,resistin,adiponectin,leptin,CRP,Prealbumin,insulin,IGF-1	TSH, AST, creatinine, WBC or lymphocytes, creatine
Zhang, D. et al., 2009[233]	Comparative and functional genomics	China	Cachexia (n=61), Weight stable (n=64), total (n=125)	Gastric	~50's	M &F	Not in the 4 weeks before assessment	Weight loss: self reported	(1) weight loss (10% of their pre illness stable weight within 6 months), (2) low food intake (1500kcal/d	N/A	Serum	IL-8 (total levels and +781 TT genotype),albumin	

									), (3) systemic inflammation (CRP 10mg/L)				
Bo, S. et al., 2010[234]	Journal of interferon and cytokine research	China	Cachexia (n=96), Weight stable (n=112), total (n=208)	Gastric	Late 50's-60's	M &F	Not in the 4 weeks before assessment	Weight loss: self reported	>10% weight loss in 6 months	N/A	Serum	Il-8 (total levels and +781 TT genotype) A251 T781 IL-8 haplotype , albumin	
Sun, F. et al., 2010[235]	Annals of clinical and laboratory science	China	Cachexia (n=107), Weight stable (n=116), total (n=223)	Gastric	~60	M &F	Not in the 4 weeks before assessment	Weight loss: self reported	weight loss was >10% of their pre-illness stable weight within 6 months and if their serum CRP level was >10 mg/L	N/A	Serum	IL-10 (Total levels and 1082AG and 819CC genotypes, GCC Haptlotype)	
Sun, F. et al., 2010[236]	Molecular diagnosis and therapy	China	Cachexia (n=85), Weight stable (n=105), total (n=190)	Gastric	60-70	M &F	Not in the 4 weeks before assessment	Weight loss: self reported	weight loss was >10% and their CRP level was >10mg/L	N/A	Serum	il-10 (Total levels and IL10 -1082G allele and -1082AG and -819CC genotypes), albumin, CRP	
Jiang, Y. et al., 2014[237]	Journal of interferon and cytokine research	Qingdao, China	Cachexia (n=39), Weight stable (n=87), total (n=126)	Gastric	~60	M &F	Pre-surgery and chemotherapy/radiation	Weight loss: self reported	weight loss >10% of their pre-illness stable weight within 6 months and serum CRP>10 mg/L.	N/A	Serum	CRP, albumin	IL-6, TNFα, IFNY
Kojima Y, et al., 2023[101]	Nature communications	Nagoya, Japan	Cachexia (n=27), Weight stable (n=19), total (n=46)	Gastric	~60s	M &F	Not stated	Weight loss: self reported	Fearon et al., [2]	N/A	Blood	Choline and tryptophan performed the best out of the metabolites shown to predict cachexia, crp + albumin	
de Castro GS, et al., 2021[111]	Clinical Nutrition	Sao Paulo, Brazil	Cachexia (n= 62), Weight stable (n=32), total (n=94)	Gastric and Colorectal	~61 (30-90)	M &F	Pre-surgery	Skeletal muscle A single axial CT image at the third lumbar vertebra , Muscle density: HU, Weight loss: scales	Evans et al.,[34]	ESPEN guidelines for nutrition in cancer patients:men < 55 cm2 /m2 and woman < 39 cm2 /m2	Plasma + serum	low LDL and HDL cholesterol, haemoglobin, High CRP, IL-6, IL-8, FABP3	FSTL-1,BDNF,IL-15,albumin,TNFa

Han J, et al., 2018[96]	Lipids in health and disease	Shanghai, China	Weight stable (n=50), early-stage cancer cachexia (n=40), and late-stage cancer cachexia (n=28), total (n=118)	Gastric and colorectal	~60's (Late stage cachexia ~59)	M &F	Pre-surgery and chemotherapy/radiation	Weight loss: not reported	Fearon et al., and late-stage cachexia defined as patients with weight loss >10% in the past 6 months	N/A	Serum	IL-6, TNFα, albumin (late stage cachexia), Free fatty acids	Triglycerides
Diakowska, D et al., 2014[135]	Disease Markers	Wroclaw, Poland	Cachexia (n=44), Weight stable (n=41), Healthy controls (n=60), total (n=145)	Gastric and oesophageal	~60	M &F	Pre-surgery and chemotherapy/radiation	Weight loss	weight loss exceeding 5% of previous baseline body weight during three-month period	N/A	Serum	Resistin	Haemoglobin, lymphocytes, Total protein, Albumin, HS-CRP, adiponectin, Apelin (Most factors different to healthy controls but not non-cachectic patients)
Krzystek-Korpacka, M. et al., 2008[203]	Clinical chemical laboratory medicine	Poland	Cachexia (n)=49, Weight stable (n=47), Healthy control (n=42), total (n=138)	Gastric and oesophageal	~59 (35-85), healthy ~34 years (25–56)	M &F	Pre-surgery and chemotherapy/radiation	Weight loss: self reported	5% during 3 months	N/A	Serum	CRP, Transferrin	IL-6, IL-8, cVEGF-A and TNFα
Deans, D.A.C. et al., 2009[89]	The American journal of clinical nutrition	Scotland	Cachexia (n=124), Weight stable (n=79), total (n=203)	Gastric and oesophageal	Average 71 (62–78)	M &F	Pre-surgery and chemotherapy/radiation	BMI, weight loss (self reported),midarm circumference and triceps skinfold thickness (Harpenden skin calipers), midarm muscle circumference	Weight loss >5% since diagnosis	N/A	Serum	IL-10 –1082 polymorphism, CRP, IL-8 and sTNF-r	il-6

Skipworth RJ, et al., 2010[99]	British Journal of cancer	Country not stated but authors are based in Scotland and Australia. Ethics granted by scotish based committee	Cachexia (n=~100, authors state 34% of 293 cancer patients lost 10% body weight), Weight stable (n=~193), healthy controls (n=35), total (n=328)	Gastric and oesophageal	~70 (26-91)	M &F	Pre-surgery and chemotherapy/radiation (Blood taken at diagnosis)	Weight loss: self-reported, BMI, Mid-arm muscle circumference, Triceps skin fold thickness	Cachexia was defined as weight loss of ?10% when compared with pre-morbid weight	N/A	Plasma	MIC-1 (Not correlated with weight loss but higher in those with >10% weight loss),CRP	
Malgorzata Krzystek-Korpacka, et al., 2007[108]	Clinical biochemistry	Wroclaw, Poland	Cachexia (n=49), Weight stable (n=47), healthy control (n=42), total (n=138)	Gastroesophageal	~59 (35-85)	M &F	Pre-surgery and chemotherapy/radiation	Weight loss: self reported	Weight loss >5% in 3 months	N/A	Serum	IL-6,IL-8, Midkine (significant in ROC analysis and has a p-value of 0.053 in t-test between cachexia patients vs non-cachexia patients)	Il-1,Tnfα, VEGF-A,VEGF-C
Guo-Tian Ruan, et al., 2023[95]	Journal cachexia, sarcopenia, muscle	China	Cachexia (n=344), Weight stable (n=561), total (n=905)	Colorectal	~59.3	M &F	Pre-surgery and chemotherapy/radiation	Weight loss: self reported,	Fearon et al.,[2]	N/A	Serum + blood	Albumin, lymphocytes, CRP, CRP-albumin ratio, lymphocyte to C-reactive protein ratio, lymphocyte CRP score, mGPS	
Deans, DA et al., 2009[90]	British Journal of Cancer	Edinburgh, Scotland	Weight loss (n=85), Weight stable (n=38), total (n=123)	Gastroesophaeal	~71 (62 – 78)	M &F	Pre-surgery and chemotherapy/radiation	Body weight loss (self reported), BMI	Weight loss yes/no	N/A	Serum	CRP,α1-antichymotrypsin, transferrin, haptoglobin (data only shown for CRP)	
Connelly-Frost, A. et al., 2009[102]	Nutrition and cancer	Carolina, USA	total (n=1691), correlation, no stratification.	Colon cancer	40-80	M &F	Pre-surgery	Weight loss: self reported	Correlation, no cut-off given	N/A	Serum	Selenium	
Zhang, S.-S. et al., 2016[97]	Oncotarget	Guangzhou, China	Weight loss (n=682), Weight stable (n=830),	oesophageal cancer	~58	M &F	Pre-surgery and chemotherapy/radiation	Weight loss	Weight loss, yes/no. No cut-off given	N/A	Serum	Fibrinogen	

			total (n=1512)										
Florescu, A. et al., 2019[107]	PLOS one	Iasi, Romania	Weight loss (n=25), Weight stable (n=34),total (n=49)	Rectal	~60's	M &F	Pre-surgery	Weight loss: self reported, current weight: scale	No cut off given	N/A	Serum	Adiponectin (women only)	Leptin
Mao, Y. et al., 2023[91]	Frontiers in Oncology	Guangxi, China	Weight loss (n=203), weight stable (n=425), total (n=628)	Gastric	~50-60 (45-67)	M &F	Pre-surgery and chemotherapy/radiation	Weight loss, BMI	patients who lost more than 2.4% of their involuntary weight within six months were considered to have weight loss	N/A	Serum	HS-CRP	
Puhr, H.C. et al., 2023[94]	Journal of Cancer research and clinical oncology	Vienna, Austria	Weight loss (n=~299), Weight stable (n=~390) (graphed, not explicitly stated as weight loss was not the main outcome)	Gastric and oesophageal	~63.9	M &F	Pre-surgery and I believe pre-treatment	Weight loss, BMI	Weight loss yes/no	N/A	Blood	neutrophil/lymphocyte ratio, Leucocyte-to-lymphocyte ratio, platelet-to-lymphocyte ratio, systemic inflammation response index , mGPS	
Daniele, A. et al., 2017[85]	Anticancer research	Italy	Malnourished (n=19, at risk of malnutrition (n=36), well nourished (n=23) healthy control (n=30), total (n=108)	Colorectal cancer	~78 (44-87)	M &F	I believe patients are pre-surgery but not 100%, stated as "undergoing" surgery.	Fat free mass and fat mass (FM) was carried out according to Durnin-Womersley scheme (Progeo s.r.l.2012-2015-Italy), measuring 4 folds: biceps, triceps, subscapularis,and suprailliac by	MNA assessment, Malnourished patients in this study had>10% weight loss in 6 months, reduced muscle mass, severe reduction in food intake due to loss of appetite and altered taste perception	Reduced muscle mass is not well outlined except for :more than 10% less than the normal range	Serum	Haematocrit, transferrin, albumin, CRP, IL-6, TNFα	

								plicometer FAT-1 (GIMA s.r.l.-Italy)					
Prokopchuk O, et al., 2021[112]	Journal cachexia, sarcopenia, and muscle	Munich, Germany	Weight loss (n=34), weight stable (n=48), total (n=82)	Colorectal cancer	~70	M &F	pre-surgery	Weight loss: scales	>5% weight loss in 6 months	N/A	Plasma	Timp-1	
Ningzhe Shen et al., 2023[84]	Journal of gastrointestinal Oncology	Wenzhou, China	Malnourished (n=118), Not malnourished (n=267), total (n=385)	Colorectal	~70	M &F	Pre-chemo/radiotherapy and I believe measurements were taken prior to surgery as they say baseline characteristics	Weight loss, Handgrip strength, BMI	Used GLIM criteria, as all had CRC the authors decided to call all patients with one of the following criteria as malnourished (I) non-volitional weight loss: weight loss >5% within the past 6 months or >10% beyond 6 months; (II) low BMI: <18.5 if <70 years old or <20 if ≥70 years old; and (III) reduced muscle mass: HGS <26 kg for men and <18 kg for women	N/A	Serum	albumin, haemoglobin	
Chiang HC, et al., 2022[98]	Nutrition and cancer	Not reported but authors are from Taiwan.	Cachexia (n=41), Weight stable (n=51), total (n=92)	oesophageal squamous cell carcinoma	~57.1	M &F	Not Pre-surgery and chemotherapy/radiation	Weight loss: self reported	weight loss of more than 5% over the past 6 mo. Called "malnutrition" in this study	N/A	Serum	Pre-albumin, transferrin (ROC analysis showed it to be a good predictor but it was not significant in a direct comparison between groups)	Albumin

Pan YP et al., 2015[136]	Asia pacific journal of clinical nutrition	Keelung, Taiwan	Cachexia (n=38), Weight stable (n=126), total (n=164)	Colorectal cancer	~60s (18-94)	M &F	Pre-surgery, not stated if other treatments were received	Weight loss, BMI	Weight loss>5%, BMI <18.5	N/A	Serum	weight loss: Trend towards significance in White blood cells, albumin, glutamine. Low BMI: albumin	CRP, arginine, carcinoembryonic antigen, total lymphocyte count, Haemoglobin, aspartate aminotransferase
Nakajima, T.E. et al., 2010[106]	Journal of Cancer research and clinical oncology	Tokyo, Japan	Cancer patients =117, matched controls =117. patients not stratified as BMI was used for correlation	Esophageal	~63.6	M &F	Pre-surgery and chemotherapy/radiation	BMI	BMI correlated with parameters.	N/A	Blood	Adiponectin, Leptin weakly correlated with BMI.	Resistin, Visfatin
Zhang, Y. et al., 2022[109]	Nutrition and cancer	Beijing, China	At risk of malnutrition (n=28), not at risk of malnutrition (n=38), total (n=66)	Gastric	~60.5	M &F	Pre-surgery	Skeletal muscle, fat mass, fat mass percentage, and fat-free mass: BIA + CT	N/A	low appendicular skeletal muscle mass was defined as < 7 kg/m2 for males and < 5.7 kg/m2 for females, and low Fat free mass index was defined as < 17?kg/m2 in men and < 15?kg/m2 in women. Fat mass index < 7.7 kg/m2 in men and < 5 kg/m2 in women were considered as having low FMI	Plasma	FGF21 (difference caused by low fat mass but not muscle mass)	

Supplementary Table 2: Included studies that identified biomarkers of sarcopenia

Reference	Journal	Location	n number	Cancer type	Age	Sex	Treatment status of cohort:	Body composition measurements	Sarcopenia definition/cut off for low SMI handgrip etc	Cut-off men	Cut-off women	Sample type	Biomarker	Biomarkers examined that had no difference between groups
Sakai, M. et al., 2021[238]	The American journal of surgery	Maebashi, Japan	Pre-sarcopenia (n=49), No pre-sarcopenia=40, total (n=89)	Oesophageal	~60s	M&F	Pre-surgery and chemotherapy/radiation	Skeletal muscle: 3rd lumbar vertebra	Pre-sarcopenia (SMI)	52.4 cm^2/m^2	35.5 $cm2/m^2$	Blood	neutrophil/lymphocyte ratio	
Shinsyu, A. et al., 2020[125]	Oncology Letters	Kusatsu, Japan	n=51, no stratification, correlation used	Oesophageal, gastric, colorectal	~64	M&F	Patients had not received chemo or radiotherapy in the last month. Had not had surgery	Fat free mass: BIA, Body fat mass: BIA, percent triceps skin fold thickness, percent arm muscle circumference, Percent ideal body weight, body weight, BMI	Fat free mass as measured by BIA is correlated with inflammatory markers but a low FFM vs High FFM is not given			Serum	TNF-α	IL-6 (just missed significant cut-off (p-value=0.06) for a correlation with FFM, Active ghrelin, leptin. IL-6 correlated with a ratio of resting energy expenditure /FFM but this only applied to stage I-II
Okugawa Y, et al., 2019[130]	Journal cachexia, sarcopenia, and muscle	TSU, Japan	Low Psoas muscle mass index (n=71), high Psoas muscle mass index (n=112) total (n=183)	Colorectal cancer	~68 (35-89)	M&F	Pre-surgery and chemotherapy/radiation	psoas muscle mass index: 4th lumbar vertebra, Intramuscular adipose tissue content : mean CT value of ROI of multifidus muscle (HU)?mean CT value of ROI of subcutaneous fat HU	Low PMI: males (AUC = 0.633) and females (AUC = 0.567)	5.896 cm^2/m^2	4.067 cm^2/m^2	Serum	miR-203, haemoglobin, albumin	

He, W.-Z. et al., 2018[123]	Cancer management and Research	Guangzhou, China	Sarcopenia (n=280), No sarcopenia (n=281), total (n=561)	Colon cancer	~59 (19-87)	M&F	Pre-surgery and chemotherapy/radiation	Skeletal muscle CT 3rd lumbar vertebra	Patients were grouped into low and high SMI according to the median SMI values for men and women in this study.			Serum	Neutrophil, lymphocyte, monocyte, platelets, Leukocyte, albumin, neutrophil/lymphocyte ratio, increased interferon-induced protein 10 (ip-10)	CRP, carcinoembryonic antigen, IL-1?, IL-1?, IL-1RA, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-13, IL-15, IL-17, IL-12 P40, IL-12 P70, EGF, eotaxin, G-CSF, GM-CSF, IFN?-2, IFN?, MCP-1, MCP-3, MIP-1?, MIP-1?, TNF?, TNF?, VEGF, FGF-2, TGF-?, FIT-3L, fractalkine, GRO, MDC, sCD40L, and sIL-2R?
Takano, Y. et al., 2023[131]	Surgery today	Tokyo, Japan	Sarcopenia (n=118), No Sarcopenia (n=113), total (n=231)	Colorectal cancer	~73 (23–98)	M&F	Pre-surgery	Skeletal muscle CT 3rd lumbar vertebra	Sarcopenia was defined as a low SMI	43.75 cm^2/m^2	41.10 cm^2/m^2	Serum, blood	Cholinesterase	.
Dolan RD, et al., 2019[239]	Journal cachexia, sarcopenia, and muscle	Scotland	Percentage of patients with sarcopenia changes with definition. Martin et al : Sarcopenia (n=283), No sarcopenia (n=367) total (n=650)	Colorectal	Majority >65	M&F	Pre-surgery	Skeletal muscle: CT scan 3rd lumbar vertebra. visceral fat area, SCA, Skeletal muscle density	Multiple definitions used: Martin et al, Caan et al., Dolan et al.,	See paper for details	See paper for details	Serum	mGPS (Albumin, CRP) significant for sarcopenia and low SMD	neutrophil lymphocyte ratio, neutrophil-platelet score

Zhang, Y. et al., 2023[126]	Frontiers in oncology	Yantai, China	Training cohort (Sarcopenia (n=55), No sarcopenia (n=232) Validation cohort (Sarcopenia (n=14), No sarcopenia (n=58))	Colorectal	60-70	M&F	Pre-surgery	Skeletal muscle: BIA, Handgrip: grip strength meter.	AWGS 2019: male grip strength < 28.0kg and female grip strength < 18.0kg, < 1.0m/s is the diagnostic cut-off value of physical decline.	7.0kg/m^2	5.7kg/m^2	Not stated but assumed to be serum due to albumin being measured in serum. Cells isolated from blood directly	Total protein, albumin, calcium, uric acid, creatine kinase, haemoglobin, neutrophil/lymphocyte ratio	
Miyamoto, Y. M. et al., 2015[129]	Annals of surgical oncology	Kumamoto, Japan	Sarcopenia (n=55), No sarcopenia (n=165), total (n=220)	Colorectal	~60-70 (30-93)	M&F	Pre-surgery	Skeletal muscle CT scan 3rd lumbar vertebra	The lowest sex specific skeletal muscle mass quartile was defined as Sarcopenia	32.6-49.5 cm^2/m^2	15.6-42.1 cm^2/m^2	Serum	carcinoembryonic antigen	
Aro, R. et al., 2022[137]	Journal of clinical medicine	Oulu, Finland	Sarcopenia (n=76), No Sarcopenia (n=79), Myosteatosis (n=20), Myosteatosis + Sarcopenia (n=47), total (n=222)	Colorectal	~70	M&F	Pre-surgery and chemotherapy/radiation	Skeletal muscle CT 3rd lumbar vertebra,	Martin et al.:	43 cm^2/m^2, for BMI < 25 kg/m2 and 53 cm^2/m^2 for BMI 25 kg/m^2	41 cm^2/m^2	Serum, blood	neutrophil/lymphocyte ratio, albumin	CRP, mGPS, IL-1R1, IL-4, IL-6, IL-7, CXCL8, IL-9, IL-12p70, IFNG, CXCL10, CCL2, CCL4, CCL11, and PDGF-BB
Guthrie, G.J.K. et al., 2013[240]	British Journal of cancer	Glasgow, Scotland	Patients used for correlation with IL-6: low SMI (n=9), Medium SMI (n=16), high (n=20), total (n=45)	Colorectal	Majority >65	M&F	Pre-surgery	Skeletal muscle CT 3rd lumbar vertebra	Sex specific quartiles based on Richards et al.,	42 cm^2/m^2	32.5 cm^2/m^2	Serum	IL-6	.
Richards, C.H. et al., 2012[116]	PLOS one	Glasgow, Scotland	Low SMI (n=59), Medium (n=59), High (n=56), total (n=174)	Colorectal	~60-70's	M&F	Pre-surgery	Skeletal muscle CT 3rd lumbar vertebra, Total fat, subcutaneous fat, visceral fat. BMI	Sex specific quartiles Men:	42 cm^2/m^2	32.5 cm^2/m^2	Serum	mGPS, CRP, albumin, Anaemia	white cell count, neutrophil/lymphocyte ratio

Ren, Q. et al., 2024[128]	Nutrition	China	Training cohort = 1353 (Low SMI (n=410), Normal SMI (n=943) Validation cohort (n=339 (Low SMI (n=98), Normal SMI (n=241)	Colorectal	~late 60's-early 70's	M&F	Pre-surgery and chemotherapy/radiation	Skeletal muscle: CT 3rd lumbar vertebra, Skeletal muscle density, muscle strength: hand dynamometer	Sarcopenia =Low muscle mass. L skeletal muscle density: sex-specific cutoff values were determined: <28.6 HU for female and <38.5 HU for male participants.	40.8 cm^2/m^2	34.9 m^2/m^2	Serum	Haemoglobin (Training but not validation), Albumin is different between those with low Skeletal muscle density vs normal Skeletal muscle density	
Souza BU, et al., 2018[11]	Nutrition and cancer	Rio de Janeiro, Brazil	Sarcopenia (n=29), No sarcopenia (n=166), total (n=195)	Colorectal cancer	~60.5	M&F	57% of patients had already undergone treatment	Skeletal muscle CT image at 3rd lumbar vertebra, body weight: scale, BMI, weight loss: self-reported, Muscle density: HU, Handgrip strength: Jamar hydraulic hand dynamometer, walking: 4.6 m gait speed test	EWGSOP: Pre-sarcopenia: low skeletal muscle mass sarcopenia: low skeletal muscle mass + low muscle strength and/or low physical. Martin et al., definition of low muscle mass	43 cm^2/m^2 for BMI < 25 kg/m^2 and 53 cm^2/m^2 for men with BMI 25 kg/m^2	41 cm^2/m^2	Serum	Albumin	CRP
Okugawa Y, et al., 2018[115]	Journal of parenteral and Enteral nutrition	Tsu, Japan	Sarcopenia (n=155), Not sarcopenia (n=153), total (n=308)	Colorectal cancer	~60s	M&F	Pre-surgery and chemotherapy/radiation	psoas muscle mass index: CT of 4th lumbar vertebra, Intramuscular adipose tissue content : HU	Low psoas muscle index	240.8	215.9	Serum	CRP, systemic immune-inflammation index (SII), neutrophil-platelet score, lymphocyte-monocyte ratio, serum albumin	

Malietzis, G. et al., 2016[241]	Tumour biology	London, England	Sarcopenia (n=14), Sarcopenia + obesity (n=4), No sarcopenia (n=3), (n=21)	Colorectal cancer	~70	M&F	Pre-surgery, Not receiving neoadjuvant chemotherapy	Skeletal muscle CT scan 3rd lumbar vertebra, SAT, VAT, Muscle attenuation	Uncertain if Prado or Martin definition was used as they linked both but didn't say which one			Blood	CD40 dendritic cells (correlation with SMI)	CCR-7,CD36,CD36M,CD83,CD83 m
Oflazoglu, U. et al., 2022[117]	European geriatric medicine	Izmir, Turkey	Sarcopenia (n=25), No sarcopenia (n=25), total (n=50)	Colorectal cancer	~60 years (range: 21–81)	M&F	Pre-surgery and chemotherapy/radiation	Skeletal muscle BIA, handgrip: dynamometer, BMI	EWSGOP: sarcopenia: low muscle mass + low muscle strength and/or low physical performance. Low handgrip strength men:<27 kg, women: <16 kg	10.76 kg/m^2	6.76 kg/m^2	Serum	irisin and FGF21, CRP	
McSorley, S.T. et al., 2018[242]	Clinical Nutrition	Scotland	Prado (Sarcopenia (n=158, No sarcopenia (n=164), Martin (Sarcopenia (n=152), No sarcopenia (n=170), total (n=322)	Colorectal cancer	Majority >65	M&F	Pre-surgery	Skeletal muscle, visceral fat area and subcutaneous fat area: 3rd lumbar vertebra	Prado et al., Martin et al., Myosteatosis was defined by SMD <41 HU in patients with BMI <25 kg/m2 and <33 HU in patients with BMI >25 kg/m2	Prado: <52.4 cm2/m Martin: 43 cm^2/m^2 for BMI < 25 kg/m^2 and 53 cm^2/m^2 for men with BMI 25 kg/m^2	Prado: <38.5 cm2/m2 Martin: 41 cm^2/m^2	Serum	mGPS (albumin, CRP)	
van Dijk DPJ, et al., 2018[114]	Journal cachexia, sarcopenia, and muscle	Calgary, Canada	Low SMI (n=60), high SMI (n=37), total (n=97)	Colorectal cancer (with liver Mets)	~60s	M&F	Pre-surgery	Skeletal muscle (SM) CT scan 3rd lumbar vertebra, Visceral, subcutaneous fat (VAT, SAT) analysed using same image. Skeletal muscle density(SMD)	Martin et al.	43 cm^2/m^2	41 cm^2/m^2	Serum	CRP	
Lv Y, et al., 2019[138]	Aging	Shanghai, China	Low SMI (n=309), normal SMI (n=230), total (n=539	Colorectal cancer (with liver Mets)	~60s (24-92)	M&F	pre-surgery	Skeletal muscle CT scan, vertebra location not	Low SMI	43 cm2/m2	41 cm2/m2	Serum	Pre-albumin	transferrin, CRP, lymphocyte count, carcinoembryonic

								mentioned						antigen and CA19-9
Fukushima T, et al., 2023[118]	Surgery Today	Kashiwa, Japan	Moderate sarcopenia (n=166), severe sarcopenia (n=38), No sarcopenia (n=70), total (n=274)	Oesophageal	60's (58-71)	M& F	Pre-surgery but most received chemotherapy or chemoradiotherapy	Skeletal muscle CT scan 3rd lumbar vertebra, BMI, handgrip strength (dynamometer)	AWGS 2019: moderate sarcopenia was defined as (1) low skeletal muscle mass or (2) low handgrip strength and/or low gait speed. Severe sarcopenia was defined as a low skeletal muscle mass, low muscle strength, and low physical performance.	52.4 cm^2/m^2	38.5 cm^2/m^2	Serum	CRP (higher in severe sarcopenia),haemoglobin	
Watanabe, A. et al., 2022[127]	Annals of surgical oncology	Kobe, Japan	Sarcopenia (n=33), No sarcopenia (n=98), total (n=131)	Oesophageal	~late 60's	M& F	Pre-surgery	Skeletal muscle CT scan 3rd lumbar vertebra, SMI + Psoas muscle index	AWGS: Sarcopenia: Low skeletal muscle + low muscle strength and/or low physical performance. To assess muscle strength, grip strength of <26 kg for males and <18 kg for females was defined as low muscle strength. Low physical performance was defined as a walking speed slower than 0.8 m/s. They then divided people into actual sarcopenia and non actual sarcopenia. Actual sarcopenia	7.0 kg/m^2	5.7kg/m^2	Serum	Men with sarcopenia are higher in the group with low Creatine x albumin	.

									was diagnosed as a sarcopenia diagnosis + low creatine x albumin.					
Tan, X. et al., 2021[122]	Cancer management and Research	Nanning, China	Patients divided into quartiles, total (G1 (lowest) -G4 (highest)), G1 (n=38), G2 (n=41), G3 (n=41), G4 (n=38), total (n=189)	Oesophageal	~70.7 (65-86)	M&F	Pre-surgery and chemotherapy/radiation	Skeletal muscle CT scan 3rd lumbar vertebra, BMI: LOW>18.5	Low L3SMI vs High L3SMI based on quartiles of the group. ROC analyses used to determine which quartile served as the cut-off. Q1 is defined as low SMI while the other quartiles are high SMI.			Serum	Neutrophil, lymphocyte ratio, prognostic nutritional index (PNI): serum albumin concentration and the total number of peripheral blood lymphocytes. GNRI: albumin * body weight. However albumin alone wasn't correlated.	Albumin
Matsunaga, T. et al., 2019[139]	Anticancer research	Osaka, Japan	Sarcopenia (n=82), No sarcopenia (n=81), total (n=163)	Oesophageal	~60s	M&F	Pre-surgery	Skeletal muscle: BIA, BMI, handgrip strength	Sarcopenia was defined as a Skeletal muscle mass (SMM) below the lower limit of the standard SMM			Not stated but assumed to be serum due to albumin being measured in serum. Cells isolated from blood directly	CRP/albumination, mGPS, platelets	CRP, albumin, lymphocytes, neutrophils, WBCS
Liang, H. et al., 2021[243]	Cancer management and research	Nanning, China	Sarcopenia (n=77), No sarcopenia (n=23), total (n=100)	Oesophageal	~59 (41-84)	M&F	Pre-surgery and chemotherapy/radiation	Skeletal muscle CT 3rd lumbar vertebra, BMI	Sarcopenia: low SMI	52.4 cm^2/m^2	38.5 cm^2/m^2	Blood	lymphocyte–monocyte ratio	platelet-lymphocyte ratio, neutrophil-lymphocyte ratio

Nambara M, et al., 2021[120]	World Journal of Surgery	Osaka, Japan	Sarcopenia percentage differed based on the definition used but the modified EWGSOP had: sarcopenia (n=22), no sarcopenia (n=51), total (n=73)	oesophageal cancer	~62 (38-82)	M&F	Neoadjuvant chemotherapy but measurements were taken pre-surgery	Skeletal muscle BIA, BMI	EWGSOP, AWGS, modified, <90% less than standard criteria, standard EWGSOP (Comparison of all methods)	EWGSOP: 8.87 KG/M^2 AWGS: 7.0 Kg/M^2, <90% less then standard criteria, Modified EWGSOP: 9.53	EWGSOP: 6.42 Kg/M^2 AWGS: 5.7 Kg/M^2 Kg/M^2 <90% less then standard criteria, Modified EWGSOP: 6.42 Kg/M^2	Serum	Albumin	CRP
Zhao, A. et al., 2023[244]	Frontiers in oncology	Yantai, China	Low muscle mass (n=29), high muscle mass (n=107), total (n=136)	Gastric	~62 (38-81)	M&F	Pre-surgery and chemotherapy/radiation	Skeletal muscle CT scan 3rd lumbar vertebra, BMI	In this study, the lowest quartile was considered as the cut-off point for determining the presence low muscle mass	40.6 cm^2/m^2	30.5 cm^2/m^2	Serum	Albumin	
Tamura, T. et al., 2019[140]	Anticancer research	Osaka, Japan	Sarcopenia (n=24), No sarcopenia (n=129), total (n=153)	Gastric	~70's (32-83)	M&F	Pre-surgery	Skeletal muscle BIA	Muscle mass index (MMI) value of one standard deviation or more below the gender-specific mean MMI.	15.44 kg/m^2	13.33 kg/m^2	Serum	Albumin	CRP
Ozcan, S.G.G. et al., 2023[121]	Journal of the Brazilian medical association	Not stated but authors are from Turkey	Sarcopenia (n=88), No sarcopenia (n=87), total (n=175)	Gastric	~60'S	M&F	Pre-surgery and chemotherapy/radiation	Skeletal muscle CT scan 3rd lumbar vertebra	Sarcopenia: low SMI	34.7±8.5 cm^2/m^2	29.3±6.51 cm^2/m^2	Serum	Haemoglobin, albumin, lymphocytes	neutrophil values, neutrophil/lymphocyte ratio, and PLR.

Zhou, C.-J. et al., 2017[245]	Journal of surgical research	Wenzhou, China	Sarcopenia (n=69), No sarcopenia (n=171), total (n=240)	Gastric	~70's	M& F	Pre-surgery	Skeletal muscle CT scan 3rd lumbar vertebra, Handgrip strength: hand dynamometer	AWGSOP,EWGSOP: sarcopenia: low muscle mass + low muscle strength and/or low physical performance. Low handgrip strength was defined as <26 kg for men and <18 kg for women, low physical performance was 6-meter usual gait speed <0.8 m/s	40.8cm^2/m^2	34.9cm^2/m^2	Serum (they said plasma was used but then said serum albumin in the results section so I think this was an error)	Albumin, haemoglobin	
Li, Y. et al., 2022[124]	Nutrition	Wuhan, China	Sarcopenia (n=67), No sarcopenia (n=156), total (n=223)	Gastric	~54.46	M& F	Pre-surgery and chemotherapy/radiation	Skeletal muscle & adipose tissue: 3rd lumbar vertebra, Skeletal muscle density, BMI	Sarcopenia Low SMI Myosteatosis: Women 26.20 HU Men: 34.50	37.6 cm^2/m^2	30 cm^2/m^2	Serum/Blood	Prognostic nutritional index (Albumin + 5 x total lymphocyte count)	RDW, PLR, neutrophil/lymphocyte ratio, NPR, LMR, SII
Lidoriki I, et al., 2019[170]	Clinical nutrition ESPEN	Athens, Greece	Low SMI (n=53), high SMI (n=55) total (n=108)	Gastric and oesophageal	~63.6	M& F	Pre-surgery but some had received prior treatment	BMI, Mid-upper arm circumference: tape, Skinfold measurements: caliper, Calf circumference: measuring tape at the point of greatest circumference. Weight loss: self reported, handgrip strength: hand-held dynamometer, Skeletal muscle ct scan 3rd lumbar vertebra, Muscle density: HU	Prado et al.,	52.4 cm^2/m^2	38.5 cm^2/m^2	Serum/blood -not stated but albumin is measured in serum	Albumin	Haemoglobin, Haematocrit, Total lymphocyte count, neutrophil-to-lymphocyte ratio, platelet-to-lymphocyte ratio.

Utku Oflazoglu, et al., 2020[119]	Supportive care in cancer	Izmir,Turkey	Sarcopenia at baseline (n=30), sarcopenia after chemo (n=30), Neither =30,total (n=90)	Colorectal and gastric	~60.5 (range 27–83)	M&F	Data on markers associated with sarcopenia pre and post chemo	Skeletal muscle: BIA, BMI, handgrip strength	EWSGOP: Sarcopenia: Low skeletal muscle mass + low muscle strength and/or low physical performance. Handgrip strength (for men, < 30 kg; for women, < 20 kg)	10.76 kg/m^2	6.76 kg/m^2	Serum	IL-8 and Hs-CRP	TNFα
Jingjie Xiao, et al., 2019[156]	The American journal of clinical nutrition	California, USA	Sarcopenia (n=1383), No sarcopenia (n=1879), total (n=3262)	Colorectal	~62.6	M&F	Pre-surgery and chemotherapy/radiation	Skeletal muscle, intermuscular adipose tissue, visceral adipose tissue, subcutaneous adipose tissue, : 3rd lumbar vertebra	Sarcopenia: Low SMI Low SMD : 35.5 HU (men), 32.5 (women)	For BMI <30 kg/m^2, , SMI <52.3 cm^2/m^2 For BMI ≥ 30 kg/m^2, SMI < 54.3 cm^2/m^2	For BMI <30 kg/m^2, , SMI <38.6 cm^2/m^2, BMI ≥30 kg/m^2, SMI <46.6 cm^2/m^2	Blood	Neutrophil to lymphocyte ratio	Albumin
Malietzis G et al., 2016[246]	Annals of surgery	London, England	Sarcopenia (n=496), No sarcopenia (n=267), total (n=763)	Colorectal	~69	M&F	Pre-surgery I believe	Skeletal muscle, intermuscular adipose tissue, visceral adipose tissue, subcutaneous adipose tissue, : 3rd lumbar vertebra	Martin et al.,	<43 cm^2/m^2 for BMI <25 kg/m^2 and <53 cm^2/m^2 for BMI >25 kg/m2	41 cm^2/m^2	Serum + blood	Neutrophil to lymphocyte ratio, albumin	
Koh HH, et al., 2022[247]	Annals of surgical oncology	Gangnam,S. Korea	Number of participants with Low SMI not explicitly stated, total (n=1015)	Colorectal cancer	~70	M&F	Pre-surgery, some have had chemotherapy, some not	Skeletal muscle CT image at 3rd lumbar vertebra, BMI, skeletal muscle density: HU	Martin et al.,	<43 cm^2/m^2 for BMI <25 kg/m^2 and <53 cm^2/m^2 for BMI >25 kg/m2	<41 cm^2/m^2	Serum	ALBI score: serum albumin and bilirubin levels as follows: log10bilirubin [mol/L]×0.66)+(albumin [g/L]×0.0852). SMI is associated with ALBI score in men, not so much in	

													women. SMD associated with ALBI score in both sexes.	
Zhang, F.-M. et al., 2021[248]	The American journal of clinical nutrition	Wenzhou, China	Training cohort (Low SMI n=327, Normal SMI=729, n=1315), Validation cohort (Low SMI n=82, Normal SMI=177, n=259)	Gastric	~60-70's	M&F	Pre-surgery	Skeletal muscle CT scan 3rd lumbar vertebra, Weight loss: self reported, Muscle density	EWGSOP: low handgrip strength + low muscle mass or low muscle quality. low handgrip strength was defined as <18 kg for females and <26 kg for males according to the Asian Working Group for Sarcopenia. Cutoffs for	40.8 cm2/m2	34.9 cm2/m2	Serum	Albumin (different between low vs high SMI but not significant in the multivariable logistic regression analysis, Haemoglobin	.

Chapter 2:

Modification of the protein cargo of HDL particles in patients at risk of sarcopenia – a novel sensor to guide precision nutrition interventions

Modification of the protein cargo of HDL particles in patients at risk of sarcopenia – a novel sensor to guide precision nutrition interventions

Rianna McElroy[1], Caoileann Murphy[1,2], Rachel Byrne[3], Helen M Roche[1,4*], Fiona C. McGillicuddy[3#]

1. School of Public Health, Physiotherapy and Sport Science, UCD Conway Institute, UCD Institute of Food and Health, University College Dublin (UCD), Dublin, Ireland;

2. Rural Economy and Development Programme, Teagasc Food Research Centre, Ashtown, Dublin, D15 KN3K, Ireland.

3. Diabetes Complications Research Centre, UCD Conway Institute and School of Medicine, University College Dublin, Dublin 4, Republic of Ireland.

4. Institute for Global Food Security, Queen's University Belfast, Belfast, UK

#Corresponding Author 1: Assistant Professor Fiona C. McGillicuddy

Address: UCD Conway Institute, University College Dublin, Belfield, Dublin D04 H1W8, Ireland

Email: fiona.mcgillicuddy@ucd.ie

Telephone: +353 1 716 6769

***Corresponding Author 2:** Professor Helen M. Roche

Address: UCD Conway Institute, University College Dublin, Belfield, Dublin D04 H1W8, Ireland

Email: helen.roche@ucd.ie

Telephone: +353 1 716 6845

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Abstract

Introduction: Dietary and lifestyle interventions represent a key cornerstone of managing patients with, or at risk of, sarcopenia. However, the outcomes of these interventions rely on measures of strength and mass alone to determine health benefits. Low-grade inflammation and metabolic dysfunction are considered key mechanistic drivers of sarcopenia, and an important target for preventative interventions. Biomarkers to track improvements in low-grade inflammation however are a major challenge due to its sub-acute nature. One of the emerging biomarkers in the metabolic field is the protein cargo attached to high-density lipoprotein (HDL) particles. HDL particles are derived predominantly from the liver, a key organ central to metabolic health, and their protein composition is remarkably altered in the setting of obesity with accumulation of liver-derived pro-inflammatory proteins and loss of anti-oxidant and anti-inflammatory proteins. Furthermore, the protein cargo is extremely sensitive to dietary fat intervention with differing protein profiles evident after saturated fat consumption relative to monounsaturated fat consumption in preclinical studies. We therefore hypothesized that the HDL proteome may serve as a novel, dietary modifiable, sensor of high-risk sarcopenia.

Methods: We utilized a subset of the Nutrimal study, a dietary intervention that examined the effects of leucine ± LC n-3 PUFA on muscle mass and strength in individuals >65 at risk of sarcopenia. HDL was isolated from the serum of *n*=26 participants using fast paced liquid chromatography (FPLC). Fractions containing large- (L) and small-HDL (S) were pooled separately and purified using a lipid removal assay before processing for mass spectrometry. Analysis was performed in Perseus. Proteins present in <70% of samples were removed and those remaining were Log2 transformed. Missing values were imputed from the normal distribution. A *t*-test was used to compare participants at moderate-risk versus high-risk of sarcopenia. Paired *t*-tests were used to determine proteins significantly affected by the Control, Leucine or Leucine + LC n-3 PUFA intervention (24 weeks).

Results: The HDL of participants at high-risk of sarcopenia were enriched with ceruloplasmin, paraoxonase 1, complement C4-B, and actin, cytoplasmic 1, among others while it was depleted of several immunoglobulins, sulfhydryl oxidase 1, and vitronectin, when compared to moderate-risk participants. The HDL proteome was uniquely affected by each nutritional intervention type and included proteins belonging to metabolic (Apolipoproteins), immune (immunoglobulins, complement) and inflammatory (hemopexin, alpha-1-antitrypsin, alpha-2-HS glycoprotein) pathways. The HDL proteome was uniquely altered following Leucine + LC n-3 PUFA supplementation, including decreases in apolipoproteins, the acute phase proteins alpha-2-HS-glycoprotein, and hepatocyte growth factor activator.

Conclusion: In conclusion, the HDL proteome has potential as a biomarker of sarcopenia risk. Furthermore the HDL is modifiable by diet, potentially reflecting benefits of dietary changes before they are reflected in more physical changes such as strength and muscle mass.

Introduction

In 2021, 1 in 10 people worldwide were aged 65 years or above[249]. This number is projected to increase to 1 in 6 people by 2050 due to advancements in health care and fertility decline[249]. This demographic creates various challenges, requiring proactive measures to address the needs of an ageing population. One crucial aspect of this is the promotion of healthy ageing, defined by the World Health Organisation (WHO) as "developing and maintaining the functional ability that enables well-being in older age"[250]. This definition emphasises the importance of not just increasing life expectancy but also ensuring that older individuals can maintain their independence and a good quality of life[250]. Therefore, there is an imperative to prioritise initiatives that aim to increase "health span", ensuring that individuals can enjoy not just longer lives, but healthier and happier ones as well.

Healthy ageing faces significant challenges, one of which is the prevalence of age-related conditions like sarcopenia[251-253]. Sarcopenia is the result of life-long adverse changes in muscle that culminate in low muscle strength, low muscle mass and reduced functionality in older people[9]. Prevalence varies across studies and can be greatly affected by the definition and strength test used[254-256]. In a recent Canadian study of *n*=12,592 participants ≥65 years old, 1.4%-5.2% of men and 1.6-7.2% of women were diagnosed with sarcopenia[257] while prevalence was higher (27-37%) in Irish adults ≥65 years old attending a day hospital[255]. Sarcopenia is a major driver of both morbidity (frailty, falls, loss of independence[258-260]) and mortality (2-fold higher risk within the elderly population[261, 262]) and patients typically have longer and more frequent hospital stays[263]. The economic burden of sarcopenia is also significant with a recent UK-based study estimating that the care associated costs of a patient with sarcopenia were more than double that of a patient without sarcopenia[263, 264]. As the population ages, this will severely impact the healthcare system.

Currently, sarcopenia is primarily defined by functional tests, characterised by the loss of strength, as endorsed by the European Working Group on Sarcopenia in Older People 2 (EWGSOP2)[36]. A widely used screening tool, the SARC-F score, evaluates strength, mobility (including walking, rising from a chair, climbing stairs), and fall history, serving as an initial step in identifying individuals requiring further assessment[265]. However, its sensitivity is limited and is reliant on sufficient staff and space to complete the tests, posing a significant drawback[265]. In clinical practice, handgrip-strength is commonly employed due to its simplicity and correlation with overall strength[36, 266]. Other methods include assessing lower limb strength using isometric torque techniques and chair stand tests[36]. Despite their comprehensiveness, these tools and measurements encounter various challenges[267] including time constraints of staff and inter-operator subjectiveness in assessment. Given the progressive nature of sarcopenia and its association with other health conditions, regular screening is

essential to detect its onset and progression[52, 268]. Furthermore, early identification of individuals in the initial stages of sarcopenia would be highly beneficial by enabling early intervention to prevent significant muscle wasting[269-271]. However, blood-based biomarkers that move beyond 'strength-centric' approaches to sarcopenia, that can potentially identify patients 'at-risk' of sarcopenia prior to significant wasting remain an unmet need[272].

Hormonal changes[273], inflammation[274], prolonged immobilisation[275], an inactive lifestyle[276] and malnutrition[274] all contribute to sarcopenia[277, 278]. Anabolic resistance, characterised by a blunted response to a protein/amino acid stimulus is more prevalent in older adults and considered a contributing factor to the development of sarcopenia. To overcome this blunted response, older adults are currently advised to consume 1-1.2g/kg of high-quality protein per day[279], however many do not meet this recommendation[280]. In addition to quantity, protein quality is of great importance to ensure an adequate anabolic stimulus[281, 282]. A high-quality protein is easily digested and absorbed and contains essential amino acids such as leucine and isoleucine [283]. Leucine is a branched-chain amino acid that can stimulate muscle growth, postprandial protein synthesis[284] and improve the muscle response to suboptimal protein levels in older people[285]. Long chain n-3 polyunsaturated fats (LC n-3 PUFA) have also been reported to improve measures of muscle mass and strength[286, 287] and may have anti-inflammatory properties[288]. The NUTRIMAL study however did not show improvements in handgrip strength, knee extension strength, appendicular lean mass or functionality in response to 6 months supplementation with leucine alone (Leu) (*n*=38) or combined leucine and n-3 PUFA supplementation (Leu + n-3 PUFA) (*n*=38) in a randomised cohort of Irish adults (>65 years) at risk of sarcopenia[289]. It is possible that there were 'strength-independent' benefits, in particular anti-inflammatory effects, associated with the nutritional interventions that were not captured in strength-based metrics that may yield promising long-term health benefits however biomarkers to sensitively track such health benefits are lacking.

Low-grade chronic inflammation is causally implicated in the development of multiple metabolic and age-related diseases including insulin resistance[290], cardiovascular disease[291] and Alzheimer's disease [292], with recent evidence also suggesting a key role in sarcopenia[274]. There is a strong mechanistic link between metabolic dysfunction associated with sarcopenia (increased adiposity, insulin resistance, chronic inflammation and reduced muscle mass) and the development of cardiovascular disease and similarly, reduced physical activity in cardiac patients can also accelerate the process of sarcopenia[293, 294]. Precision nutrition approaches targeting sarcopenia represent a potential gateway to promote healthy ageing and longevity by preventing associated life-limiting cardiometabolic complications, however biomarkers to guide nutritional interventions remain an unmet need[295].

Within the current study we hypothesized that the protein cargo attached to circulating high-density lipoprotein (HDL) particles, may serve as a novel, nutritionally modifiable, biomarker of patients at high-risk of developing sarcopenia due to its ability to track and monitor chronic low-grade inflammation and perturbations in metabolism[296, 297]. The HDL proteome carries a diverse range of proteins (~285 have been identified) with beneficial functions such as protease inhibition, reverse cholesterol transport and regulation of inflammation[223]. However, HDL can become dysfunctional during disease conditions and carry both beneficial and unfavourable proteins[298, 299]. It is these changes that make the HDL proteome a far superior biomarker for cardiometabolic health compared to static measurements of HDL-cholesterol (HDL-C)[297, 300, 301] and a characteristic we hope to utilise in this study. The HDL proteome also differs significantly depending on the size of the particle[302]. Preclinical studies have demonstrated that the HDL proteome is enriched with pro-inflammatory proteins and depleted of antioxidant proteins in the setting of saturated fatty acid (SFA) induced obesity relative to monounsaturated fatty acids (MUFA) induced obesity[297] indicating that the HDL proteome may serve as a novel and sensitive biomarker of low-grade chronic inflammation in obesity. The changes observed within the HDL proteome were particularly sensitive to nutritional fat intake indicative that the HDL proteome may serve as a novel nutritional sensor to guide precision nutrition interventions[297]. The HDL proteome is also enriched with pro-inflammatory proteins in patients with established cardiovascular disease[303] and end-stage renal disease[304] and thus represents an important global biomarker of cardiometabolic health, and low-grade inflammation. Within the current study we hypothesized that the HDL proteome may serve as a novel biomarker of sarcopenia risk and a novel guide to deliver precision care to patients at high-risk of sarcopenia. We determined the impact of nutritional intervention with Leu ± n-3 PUFA supplementation on the HDL proteome within the Nutrimal study. We demonstrate for the first time that the HDL proteome is altered in people at highest risk of sarcopenia relative to people at moderate-risk of sarcopenia, offering a potential risk-stratification tool to identify the highest-risk people. In addition, we demonstrate that the HDL proteome is modifiable by nutritional intervention with potent and distinct changes evident within the protein cargo of the particles after leucine with and without LC n-3 PUFA supplementation, with particular effects on apolipoproteins. This study demonstrates that the HDL proteome is a novel sensitive sensor of sarcopenia risk that may be utilized as a risk stratification tool prior to significant wasting, as well as a biomarker of response to dietary/lifestyle interventions.

Methods

Participant selection and Study design:

A subset of participants was selected from the previously completed Nutrimal supplementation study[289] which was a 24-week, randomised, double-blind nutrition intervention in community-dwelling older adults ≥65 years of age at risk of sarcopenia. The Nutrimal study enrolled participants at risk of sarcopenia (*n*=107) and randomly assigned them to one of three dietary intervention groups; placebo control (*n*=31), leucine (Leu) (*n*=38) and combined leu + n3-PUFA (*n*=38) for a 24-week study period. "At risk" of sarcopenia was defined as having low muscle mass and/or low handgrip-strength. Low skeletal muscle mass was determined using the Janssen cut-offs[305], ≤6.75 kg/m^2 in women, and ≤10.75 kg/m^2 in men, while low handgrip-strength is defined as <20kg for women and <30kg for men[289]. Participants were further categorised as moderate (6.75 - 5.76 kg/m^2 in women, 10.75 - 8.6 kg/m^2 in men) or high-risk of sarcopenia (≤5.75 kg/m^2 in women, ≤8.5 kg/m^2 in men) based on their skeletal muscle index (SMI) (skeletal muscle mass (kg)/height (m^2))[289]. The Leu supplement contained 10.6g of protein, 3.1g of which was leucine. The Leu + LC n-3 PUFA contained an additional 2g of long-chain (LC) n-3 PUFA (0.8 g EPA, 1.1 g DHA). Participants were instructed to consume each supplement, which was in the form of a juice-based drink, twice a day (21.2g of protein, 6.2g of which is leucine ± 4g of LC n-3 PUFA). The Control supplement was an isocaloric, maltodextrin-based drink. To control for fat content, the Control and Leu supplement were made with seed oils. The Leu and Leu + n-3 PUFA supplement contained 209 calories each (418 calories a day), while the Control supplement contained 200 calories (400 calories a day). Full supplement breakdown has been described previously in the original paper[289].

Body weight was measured using a calibrated scale. Skeletal muscle mass and fat mass were measured using dual-energy X-ray absorptiometry (DXA) (GE-LUNAR iDXA; Aymes Medical) and Bioelectrical impedance analysis (BIA). Skeletal muscle and fat mass reported here are based on BIA measures. Handgrip-strength was measured using a hydraulic hand dynamometer (Saehan SH5001; Glanford Electronics Ltd.). Both hands were measured and the highest handgrip-strength (Kg) was used for analysis. Composite leg-strength (Leg-strength) is the sum of isometric extension, flexon and isokinetic strength which were measured using a dynamometer (Cybex NORM; Humac) on the dominant leg. Leg-strength is recorded in maximum voluntary contraction newton meters (MVC nm) For further information please see the original Nutrimal paper[289].

HDL isolation by Fast Protein Liquid Chromatography (FPLC):

Serum was mixed with 0.01 M Phosphate Buffer Saline (PBS), 1mM EDTA and injected into the GE ÄktaFPLC system (GE Healthcare) with two Superose 6 Increase 10/300 GL columns in series (Cytiva). Fractions (0.5ml) were collected and cholesterol content across fractions was determined enzymatically using fujifilms LabAssayTM Cholesterol 500Tests kit (FUJIFILM Wako Shibayagi Corporation)

HDL purification and proteomic preparation:

FPLC fractions 36-39 were pooled to create a large (L)-HDL fraction pool while fractions 40-43 were pooled to create a small (S)-HDL fraction pool (Figure 1). Lipid removal agent (LRA) (30µl of 100mg/ml reconstituted in 50mM Ammonium bicarbonate (ABC) (Sigma-Aldrich, Ireland), was added to 400µl of pooled HDL. Samples were mixed and then centrifuged at 10,000 RPM to pellet the LRA. The supernatant containing lipid free plasma proteins was removed and the remaining pellet was washed with 50mM ABC. The pellet was resuspended in 2M urea, 50mM ABC.

Trypsin digestion:

Dithiothreitol (DTT) (5mM) was added to samples to reduce the disulphide bonds in proteins and incubated for 30 minutes at 60°C. Iodoacetamide (IAA) (10mM) was added to alkylate the cysteine residues and incubated in the dark at room temperature for 30 minutes. Samples were diluted with 50µl of Ammonium bicarbonate (ABC) to reduce urea to a working concentration for trypsin digestion. Trypsin singles containing 0.5µg of trypsin (Sigma-Aldrich, Ireland), was added to each sample and incubated overnight at 37°C with shaking. Formic acid was added to a concentration of 1% to inhibit the trypsin and centrifuged for 5min at 10,000 RPM. Supernatant containing peptides was transferred to a fresh tube. Peptides were loaded onto C18 Ziptips (Merck, Millipore) and eluted in a buffer containing 70% acetonitrile and 0.1% formic acid. Samples were dried down in a Concentrator 5301 (Eppendorf) and resuspended in 3% Acetonitrile with 0.1% formic acid. Peptides were quantified by Denovix DSC spectrometer (Denovix) at 205/215nm.

Mass spectrometry:

Peptides were analysed by Dr. Caitriona Scaife in the UCD Conway proteomic core, on a Quadrupole Orbitrap (Q-Exactive, Thermo Scientific) mass spectrometer coupled to a reversed-phase NanoLC UltiMate 3000 HPLC system (Thermo Scientific). Peptides were loaded onto a C18 reversed-phase column (length: 10cm, inner diameter: 75µm) and eluted with a linear gradient 2-27% using buffer containing 0.5% acetic acid, 97% acetonitrile in 118 min at a 250 nl/min flow rate. Injection volume was 5µl. The Orbitrap was ran in data dependent mode and switched from MS to MS2 acquisition

automatically. A survey of full-scan MS spectra (m/z 300 – 1200) was obtained using the Orbitrap at a resolution of 70,000. The MS2 spectra had a resolution of 17,500. Higher-energy C-trap dissociation was used to sequentially isolate and fragment the twelve most intense ions.

Peptide search and protein identification:

Raw data from the orbitrap was analysed using MaxQuant version 2.0.1.0 which utilises the Andromeda search engine. MS/MS spectra, allowing for 2 cleavages to be missed by trypsin, was matched to the homo sapiens Uniprot database (version 1.1.0) for protein identification. The database searches utilized carbamidomethyl (C) as a fixed modification, while acetylation (protein N terminus) and oxidation (M) were considered as variable modifications. The mass spectra were analysed using the default settings of MaxQuant, specifically with a false discovery rate (FDR) of 1% at both the peptide and protein level. To generate label-free quantitative (LFQ) ion intensities for protein profiles, MaxQuant matched signals from corresponding peptides across different nano-HPLC MS/MS runs within a maximum time window of 1 minute.

Statistical analysis:

For the proteomic analysis, raw LFQ values were analysed in Perseus (version 1.6.15.0). Contaminants, proteins only identified by site and reverse database identifications were filtered from the dataset. A valid value filter of 70% in at least one group was applied. Proteins were LOG2 transformed and missing values imputed from the normal distribution. A t-test with a p-value of 0.05 was performed to compare participants at moderate-risk and high-risk of sarcopenia. A paired t-test with a p-value of 0.05 was carried out comparing baseline to post supplementation data. Results were normalised using z-score and visualised as a heat map in Perseus software. Venn diagrams of shared significant proteins were generated using the bioinformatic and evolutionary genomics webtool. Correlation analysis was carried out in GraphPad prism (version 5.01) using a Pearson correlation on normally distributed data and Spearman on non-normally distributed data as determined by Shapiro-Wilk normality test.

For patient characteristic, baseline and post supplementation data were analysed for each group using a paired t-test if the data was normally distributed and a Wilcoxon signed rank test or sign rank test was used if the data was not normally distributed. A one-way ANOVA with Bonferroni post-hoc test was performed to determine differences between groups. This test was performed on delta change values (post value-baseline value) to account for baseline differences.

Correlation analysis was performed on baseline and delta change (Δ) (pre- versus post- intervention) data. Δ was calculated by taking baseline values away from post intervention values for both HDL associated proteins (LFQ values) and clinical/biochemical data. Δ correlation analysis was performed

on proteins that were detected and baseline and post-intervention. Where an LFQ value was reported as 0, this data point was removed to avoid conflating the results. Correlation analysis was performed on a protein if it was present in at least 50% of the samples (if this was a half number than it was round down, i.e. 12.5 would become 12).

Results

Clinical and phenotypic data of participants at moderate-risk versus high-risk of sarcopenia at baseline:

A specific sub-cohort (*n*=26) of participants with moderate-risk and high-risk of sarcopenia, based on their SMI (Moderate: 6.75 - 5.76 kg/m^2 in women, 10.75 - 8.6 kg/m^2 in men, high-risk: ≤5.75 kg/m^2 in women, ≤8.5 kg/m^2 in men[289], were selected from the larger Nutrimal study[289] for HDL proteomics analysis.

Table 1 illustrates the majority of participants were male, with an average age of 68.64 ± 1.08 years in the moderate-risk group and 70.6 ± 1.49 years in the high-risk group. Based on selection, skeletal muscle mass (SMM) and SMI were significantly different between the high-risk and moderate-risk groups ($p<0.05$ and $p<0.01$ respectively). BMI was lower in high-risk subjects, albeit not significantly ($p=0.075$). Neither a traditional measure of sarcopenia, such as handgrip strength nor leg-strength were significantly different between the moderate- and high-risk groups . The C-reactive protein (CRP) levels of both groups were also similar based on traditional biomarkers. As expected, handgrip-strength was well correlated with leg-strength ($r=0.734, p<0.0001$) and SMI ($r=0.604$, $p<0.01$). The correlation between leg strength and SMI was less robust ($r=0.431, p<0.05$)

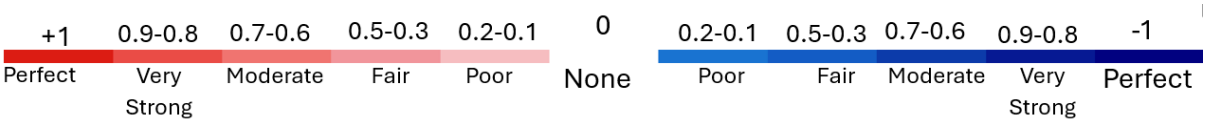
Table 1: Baseline phenotypic and clinical characteristics of participants at moderate- and high-risk of sarcopenia, based on SMI *(*Moderate: 6.75 - 5.76 kg/m^2 in women, 10.75 - 8.6 kg/m^2 in men, high-risk: ≤5.75 kg/m^2 in women, ≤8.5 kg/m^2 in men)

	Moderate-risk (*n*=11)	**High-risk (*n*=15)**	**p-value**
Sex male (n%)	9 (81.8%)	11 (73.3%)	1.00
Age (Yrs.)	68.64 ± 1.08	70.6 ± 1.49	0.464
Height (m)	1.73 ± 0.03	1.69 ± 0.02	0.329
Body mass (Kg)	81.02 ± 4.09	71.32 ± 3.31	0.075
Skeletal muscle mass (Kg)	25.33 ± 1.66	20.96 ± 1.37	0.018
Fat mass (BIA) (kg)	26.18 ± 2.54	24.56 ± 2	0.616
BMI (kg/m^2)	26.98 ± 1.04	24.8 ± 0.85	0.116
Skeletal muscle mass index (BIA) (kg/m^2)	8.36 ± 0.36	7.21 ± 0.36	0.001
Handgrip-strength (kg)	34.55 ± 2.7	31.53 ± 2.51	0.427
Leg-strength (MVC nM)	374.45 ± 23.41	358.27 ± 33.13	0.714
Total Cholesterol (mmol/L) ɬ	5.57 ± 0.33	5.83 ± 0.21	0.492
High Density lipoprotein cholesterol (mmol/L)ɬ	1.48 ± 0.08	1.63 ± 0.1	0.293
Low Density lipoprotein cholesterol (mmol/L) ɬ	3.54 ± 0.29	3.61 ± 0.22	0.848
Triglycerides (mmol/L)ɬ	1.22 ± 0.12	1.31 ± 0.16	0.645
HS-CRP (mg/L) ɬ	1.91 ± 0.32	1.55 ± 0.36	0.484
Creatinine (umol/L) ɬ	79.25 ± 6.02	78.94 ± 4.06	0.965
Glucose (mmol/L) ɬ	5.78 ± 0.2	5.53 ± 0.09	0.223
Insulin (mU/L) ɬ	5.26 ± 1.02	4.54 ± 0.55	0.515
HOMA-IR	1.4 ± 0.3	1.13 ± 0.15	0.838
Total adiponectin (µg/mL)þ	10.49 ± 2.47	9.88 ± 1.28	0.687

ɬ=measured in serum, þ=measured in plasma. Continuous data is presented as mean ± standard deviation, and categorical data is presented as n(percentage). To determine the difference between participants at a Moderate-risk and High-risk of sarcopenia, a t-test (normal data) or Mann-Whitney U test (non-normal data) was performed in SPSS.

Table 2: Correlation table of handgrip-strength, leg-strength and SMI.

	Handgrip (Kg)	Leg-strength (MVC nM)	Skeletal muscle index (Kg/m^2)
Handgrip (Kg)	1	0.734****	0.604**
Leg-strength (MVC nM)	0.734****	1	0.431*
Skeletal muscle index (Kg/m^2)	0.604**	0.431*	1



*Values displayed are correlation coefficients (r). Correlations were carried out in GraphPad Prism version 10. Pearson correlation was used for normally distributed data while Spearman correlation was used for non-normally distributed data. Significant proteins are marked with *. p*<0.05,p**<0.01,p***<0.001, p****<0.0001. Significant cells are highlighted based on the strength of the correlation. Darker red indicates a stronger positive correlation and darker blues indicate a stronger negative correlation.*

Cholesterol assay-determining large and small-HDL:

To determine which FPLC fractions contained HDL, a cholesterol assay was used to measure the cholesterol in each fraction (Figure 1). The first larger peak from fractions 22-28 contains low density lipoprotein (LDL) while the second smaller peak contains HDL. Fractions 36-39 were pooled to create a large (L) HDL sample while fractions 40-43 were pooled to create a small (S) HDL sample.

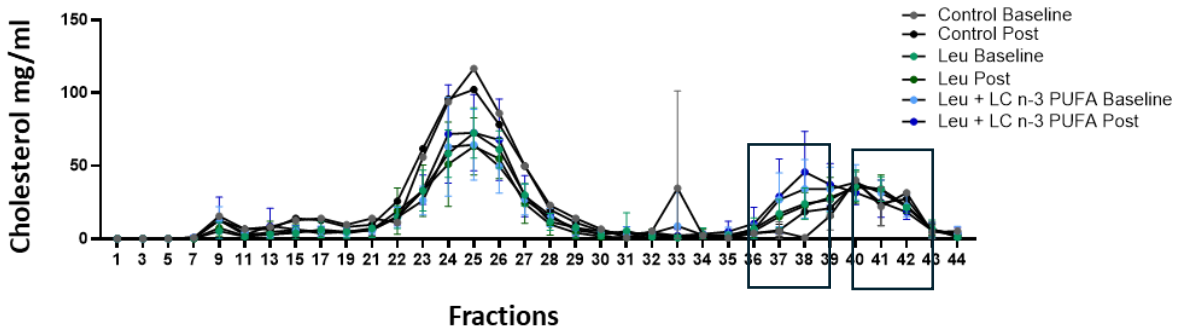


Figure 1: Cholesterol curve of FPLC fractions. Curve was used to choose L- and S- HDL. Groups are graphed individually for baseline and post intervention samples. Fractions 36-39 were pooled to create the L-HDL fraction while fractions 40-43 were pooled to create a S-HDL fraction. GraphPad prism was used to interpolate the values using a standard curve.

The proteomic composition of small (S)-HDL particles is different between participants at moderate-risk versus high-risk of sarcopenia:

We analysed the protein composition of S-HDL and L-HDL particles separately given they have different protein composition[306]. Within initial studies, we determined whether HDL proteomic composition is modulated based on measures of SMM by subdividing the combined Nutrimal subgroup (*n*=26) into moderate and high-risk of sarcopenia. Collectively, *n*=143 proteins were identified on S-HDL, 99 of which were shared with L-HDL.

When comparing moderate-risk vs. high-risk of sarcopenia, *n*=14 proteins were significantly modulated on S-HDL (Figure 2A). Of these, *n*=6 were increased in the high-risk group relative to the moderate-risk group including prothrombin (F2), complement C4-B (C4B), Keratin, type II cytoskeleton 1 (KRT1), ceruloplasmin (CP), Beta-Ala-dipeptidase (CNDP1) and serum paraoxonase 1 (PON1). A further *n*=8 proteins, exclusively immunoglobulins, were depleted on S-HDL in the high-risk group versus moderate-risk group including immunoglobulin heavy constant gamma 2 (IGHG2), Immunoglobulin heavy variable 1-18 (IGHV1-18), immunoglobulin heavy variable 3-72 (IGHV3-72), immunoglobulin kappa variable 1-17 (IGKV1-17), immunoglobulin kappa variable 3-20 (IGKV3-20), immunoglobulin

kappa variable 3D-15 (IGKV3D-15), immunoglobulin lambda constant 3 (IGLC3), and immunoglobulin lambda-like polypeptide 5 (IGLL5) (Figure 2A&B).

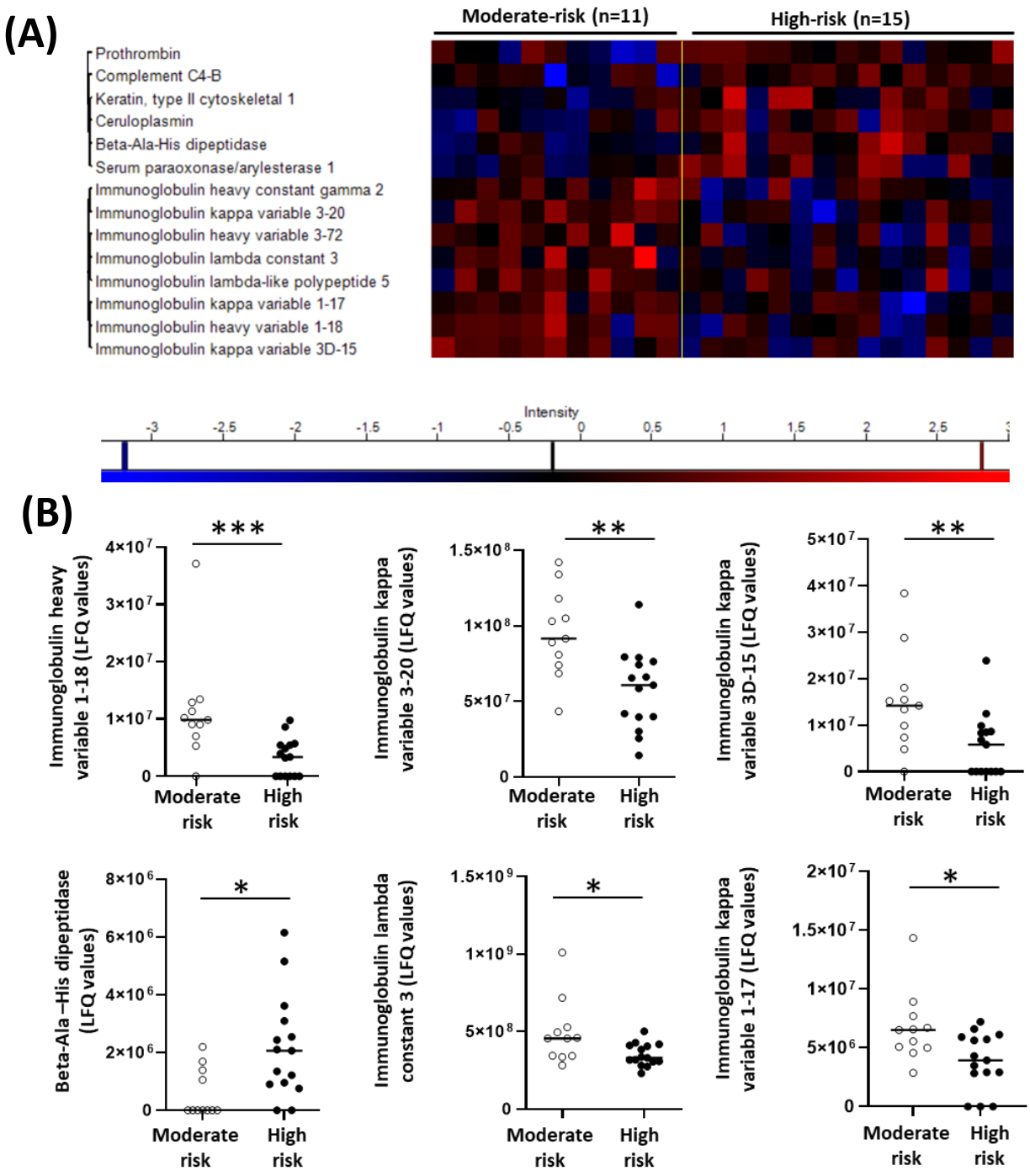


Figure 2: (A) Heatmaps and (B) dot plots of significant proteins on S-HDL particles between participants at moderate-risk versus high-risk of sarcopenia. Dot plots show the LFQ values of the top 6 most significant proteins as determined by t-test in Perseus. Significance of LFQ values was determined in GraphPad for display purposes. Moderate-risk n=11, high-risk n=15. ***p≤0.001,** p≤0.01,*p≤0.05.

The relationship between proteins associated with S-HDL particles and measures of strength and SMI prior to intervention:

To investigate the relationship between the proteomic composition of S-HDL particles and indicators of strength and muscle mass, we conducted a correlation analysis between HDL-associated proteins and measures of handgrip-strength, leg-strength and SMI at baseline in the combined cohort (*n*=26) (Table *3*).

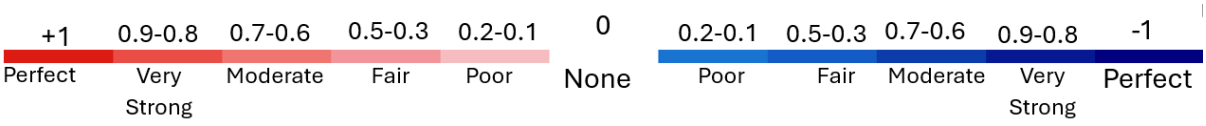
Handgrip-strength positively correlated with *n*=3 proteins and negatively correlated with *n=5* proteins on S-HDL (Table *3*). Among these, Alpha-2-HS-glycoprotein (AHSG) exhibited the highest positive association (r=0.530, p=0.0053), while plasma protease C1 inhibitor (SERPING1) displayed the most significant negative association (r=-0.628, p=0.0289). Several complement proteins on S-HDL negatively associated with handgrip-strength including Complement C1s subcomponent (C1S) (r=-0.416, p=0.0344), Complement C5 (C5) (r=-0.398, p=0.044) and complement component C6 (C6) (r=-0.403, p=0.0415), while apolipoprotein D (ApoD) positively correlated (r=0.436, p=0.0253) with handgrip strength (Table *3*).

Leg-strength positively correlated with *n*=3 proteins and negatively correlated with *n*=3 proteins. Among these, only ApoD (r=0.581, p=0.0019) was shared with handgrip-strength (Table *3*). Complement protein C4-B (C4B) (r=0.411, p=0.0411) positively correlated with leg-strength. Sulfhydryl oxidase 1 (QSOX1) had the greatest positive association (r=0.595, p=0.0017) while serotransferrin (TF) had the greatest negative association (r=-0.58, p=0.0019) with leg-strength (Table *3*).

SMI was correlated with *n*=13 S-HDL proteins. Of these *n*=8 proteins correlated positively with SMI, while *n*=5 negatively correlated with SMI. There was a positive correlation between immunoglobulins (n=7) with SMI. Immunoglobulin kappa variable 1-5 (IGKV 1-5) had the strongest association (r=0.662, p=0.0121) while SERPING1 had the strongest negative association (r=-0.741, p=0.0078) (Table *3*). Both IGHV1-18 and IGHV1-17 were found to be positively correlated with SMI and were also downregulated in the participants at high-risk of sarcopenia (Figure 2). Two proteins on S-HDL correlated with measures of both SMI and leg-strength including complement C8 alpha chain (C8A) (r=-0.448, p=0.0219 and r=-0.427, p=0.0296 respectively), and TF (r=-0.448, p=0.0219 and r=-0.58, p=0.0019 respectively). There were no proteins on S-HDL particles that correlated with measures of both SMI and handgrip-strength.

Table 3: Correlation table of handgrip-strength, leg-strength and skeletal muscle index with S-HDL associated proteins at baseline

	Handgrip-strength (Kg)	Leg-strength (MVC nM)	Skeletal muscle index (kg/m^2)
AHSG	0.53**	0.324	0.151
APCS	0.159	0.364	0.441*
APOD	0.436*	0.581**	0.091
C1S	-0.416*	-0.256	-0.138
C4B	0.163	0.411*	0.06
C5	-0.398*	-0.254	-0.143
C6	-0.403*	-0.048	-0.262
C8A	-0.362	-0.427*	-0.448*
IGFBP3	0.446*	0.264	0.157
IGHV1-18	0.049	-0.09	0.575**
IGHV1-2	0.292	0.303	0.443*
IGHV2-26	0.237	0.212	0.559*
IGKV1-17	0.125	0.041	0.598**
IGKV1-5	-0.007	0.175	0.662*
IGKV4-1	0.016	0.171	0.397*
IGLV1-47	-0.145	0.13	0.413*
KRT9	-0.266	-0.182	-0.521*
PLG	-0.24	-0.394*	-0.447*
QSOX1	0.174	0.595**	0.195
SERPING1	-0.628*	-0.346	-0.741**
TF	-0.358	-0.58**	-0.448*
TGFBI	-0.438*	-0.12	-0.145



Values displayed are correlation coefficients (r). Correlations were carried out in GraphPad Prism version 10. Pearson correlation was used for normally distributed data while Spearman correlation was used for non-normally distributed data. Significant proteins are marked with *. p*<0.05, p**<0.01,

p***<0.001. Significant cells are highlighted based on the strength of the correlation. Darker red indicates a stronger positive correlation and darker blues indicate a stronger negative correlation.

The proteomic composition of large (L)-HDL particles is different between participants at moderate-risk versus high-risk of sarcopenia:

Analysis of L-HDL particles identified a change in *n*=6 proteins between those at moderate-risk versus high-risk of sarcopenia (Figure *3* A&B). Of these, two were upregulated in the high-risk group versus moderate-risk group, actin, cytoplasmic 1 (ACTB) and albumin (ALB)) while four were downregulated, QSOX1, F2, vitronectin (VTN) and coagulation factor XIII B chain (F13B).

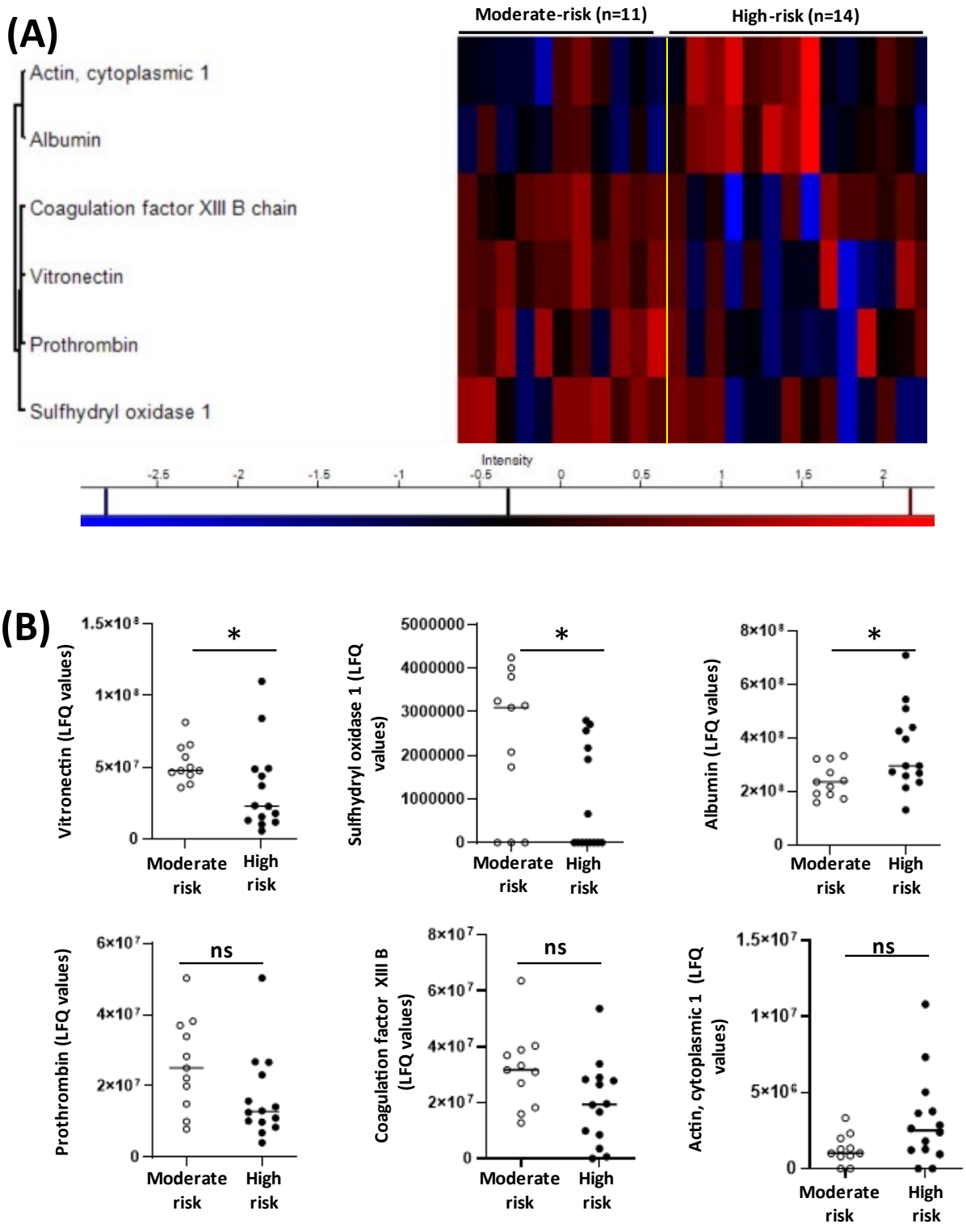


Figure 3: (A) Heatmaps and (B) dot plots of significant proteins on the L-HDL between participants at moderate-risk versus high-risk of sarcopenia. Dot plots show the LFQ values of significant proteins as determined by t-test in Perseus. Significance of LFQ values was determined in GraphPad for display purposes. Moderate-risk (n=11), high-risk (n=15). ***p≤0.001,** p≤0.01,*p≤0.05.

The relationship between proteins associated with L-HDL particles and measures of strength and SMI prior to intervention

Similar to analysis on S-HDL particles we performed a correlation analysis between L-HDL proteins with handgrip-strength, leg-strength, and SMI at baseline (Table *4*).

Leg-strength exhibited significant negative correlations with *n*=11 proteins and positive correlations with *n*=4 proteins. Inter-alpha-trypsin inhibitor heavy chain H1 (ITIH1) demonstrated the strongest negative correlation (r=-0.587, p=0.002), while Immunoglobulin kappa constant (IGKV) showed the strongest positive correlation (r=0.585, p=0.0022). Notably, the protein GSN significantly correlated with all three measurements of strength, leg-strength (r=0.551, p=0.0043), handgrip-strength (r=0.504, p=0.0101) and SMI (r=0.429, p=0.0326). SAA2-SAA4 negatively correlated with measures of leg-strength (r=-0.474, p=0.0222) and SMI (r=-0.472, p=0.0229) with a non significant correlation with handgrip-strength (r=-0.335, p=0.1184).

Handgrip-strength significantly negatively correlated with *n*=3 proteins on L-HDL particles including properdin (CFP) (r=-0.51, p=0.0183) apolipoprotein E (ApoE) (r=-0.4836, p=0.0143), complement C1R subunit (C1R) (r=-0.463, p=0.0299) and positively correlated with *n*=1 protein, Gelsolin (GSN) (r=0.504, p=0.0101) (Table *4*).

SMI negatively correlated with *n*=3 proteins including Complement factor H-related protein 1 (CFHR1) (r=-0.472, p=0.0191), apolipoprotein M (ApoM) (r=-0.447, p=0.0423) and SAA2-SAA4 (r=-0.472, p=0.0229) and positively correlated with *n*=1 protein, GSN (r=0.429, p=0.0326).

Table 4: Correlation table of handgrip-strength, leg-strength and SMI with L-HDL associated proteins

	Handgrip-strength (Kg)	Leg-strength (MVC nM)	Skeletal muscle index (kg/m²)
A2M	-0.154	-0.483*	0.168
AMBP	-0.281	-0.481*	0.01
APOA1	-0.268	-0.4*	-0.115
APOE	-0.484*	-0.241	-0.272
APOM	-0.233	-0.362	-0.447*
C1R	-0.463*	-0.389	-0.086
C4A	-0.124	-0.403*	-0.147
C5	-0.163	-0.485*	-0.162
CFHR1	-0.258	-0.326	-0.546*
CFP	-0.51*	-0.42	-0.325
FN1	-0.275	-0.516*	-0.325
GSN	0.504*	0.551**	0.429*
HGFAC	-0.267	-0.581**	-0.055
IGKV1-5	0.289	0.462*	0.295
IGHG2	0.296	0.46*	0.104
IGHG3	-0.17	-0.422*	-0.113
KRT10	0.359	0.585**	0.231
KRT9	-0.25	-0.587**	0.005
SAA2-SAA4	-0.335	-0.474*	-0.472*
SELENOP	-0.318	-0.556**	-0.16

Values displayed are correlation coefficients (r). Correlations were carried out in GraphPad Prism version 10. Pearson correlation was used for normally distributed data while Spearman correlation was used for non-normally distributed data. Significant proteins are marked with *. P*<0.05, P**<0.01,P***<0.001. Significant cells are highlighted based on the strength of the correlation. Darker red indicates a stronger positive correlation and darker blues indicate a stronger negative correlation.

Clinical and phenotypic data of participants at baseline and at 24 weeks post Leu ± LC n-3 PUFA supplementation:

Participants completed a dietary intervention, consuming additional leucine ± LC n-3 PUFA for 6 months, as part of a larger RCT[289]. Clinical characteristics of the subset of patients analysed within this study (*n*=26) are summarised in Table 5. Groups were age- and sex-matched with no significant difference in SMM between the three groups at baseline. In contrast to the isocaloric control supplement, leucine supplementation with or without LC n-3 PUFA significantly increased body weight and BMI, but had no significant impact on skeletal muscle mass or index, handgrip-strength or leg-strength, as reported previously[289]. Fat mass and HOMA-IR were significantly increased following Leu supplementation, which was not observed in the Leu + LC n-3 PUFA or control groups.. Supplementation with Leu + LC n-3 PUFA significantly decreased total adiponectin levels ($p<0.0001$) while an opposite but not significant trend was observed in the Leu alone and Control group. Plasma triglycerides were significantly decreased in the Leu + LC n-3 PUFA group ($p=0.051$).

Table 5: Phenotypic characteristics at baseline and at 24w post nutritional intervention.

	Control (n=5)[a]			Leu (n=10)[b]			Leu + fish oil (n=11)[c]			
	Pre	Post	p-value	Pre	Post	p-value	Pre	Post	p-value	p-value
Sex male (n%)	5 (100%)			8 (80%)			7 (63.6%)			0.266
Age (Yrs)	67.8±1.2	68.6±1.2	0.016	68±0.7	68.3±0.6	0.037	72.4±2	72.7±2	0.038	0.25
Height (m)	1.74±0.03			1.75±0.03			1.66±0.03			0.063
Weight (Kg)	79±3.0	78.5±3.2	0.201	78±4.6	80.3±4.3	0.002	71.5±4.6	73±4.7	0.029	a*b=0.005
Skeletal muscle mass (Kg)	24.8±1.3	24.6±1.3	0.491	25.1±1.6	25.2±2.1	0.759	21.2±2.1	22.5±2	0.46	0.561
Fat mass (kg)	26.3±2.4	26.2±2.7	0.815	25.4±3.5	28.8±3.5	0.026	24.7±2.1	26±2	0.339	a*b=0.028
BMI (kg/m2)	26±0.6	25.8±0.7	0.182	25.6±1.4	26.3±1.3	0.002	25.8±1.1	26.3±1.1	0.017	a*b=0.013
Skeletal muscle index (kg/m2)	8.1±0.2	8.1±0.3	0.532	8.2±0.3	8.1±0.4	0.929	7.5±0.5	7.9±0.5	0.429	0.147
Handgrip strength (kg)	33.8±2.8	32.6±3.4	0.185	37.7±2.5	38.1±2.7	0.696	27.9±2.9	29.3±2.2	0.836	0.617
Leg strength (MVC nM)	381.2±20.8	381.4±18.1	0.993	388.4±28.8	403.5±39.05	0.448	336.6±41.9	308.1±31.3	0.199	0.289
At high risk of sarcopenia	3 (60%)			6 (60%)			6 (54.5%)			0.962
Cholesterol (mmol/L)ŧ	6.2±0.2	5.9±0.4	0.226	5.7±0.3	5.7±0.3	0.807	5.5±0.3	5.6±0.4	0.686	0.686
HDL (mmol/L)ŧ	1.3±0.1	1.3±0	0.394	1.5±0.1	1.5±0.1	0.765	1.7±0.1	1.7±0.2	0.818	0.867
LDL (mmol/L) ŧ	4.3±0.2	4.1±0.3	0.08	3.7±0.2	3.6±0.2	0.93	3.2±0.3	3.3±0.24	0.891	0.731
Triglycerides (mmol/L)ŧ	1.4±0.2	1.5±0.4	0.72	1.2±0.1	1.5±0.2	0.084	1.3±0.2	1.1±0.2	0.051	0.051
hsCRP (mg/L) ŧ	1±0.3	1.5±0.6	0.38	1.5±0.3	2±0.4	0.182	2.2±0.5	2.4±0.5	0.735	0.9
Glucose (mmol/L) ŧ	5.7±0.2	5.7±0	0.955	5.5±0.1	5.6±0.1	0.218	5.7±0.2	5.9±0.1	0.069	0.493
Insulin (mU/L) ŧ	4.5±0.7	3.9±0.6	0.416	3.5±0.8	4±1	0.057	6.2±0.9	6.6±1.2	0.664	0.61
HOMA-IR	1.2±0.2	1±0.2	0.487	0.9±0.2	1±0.3	0.038	1.6±0.3	1.8±0.4	0.539	0.477
Total adiponectin (µg/mL)þ	11±2.6	12±2.9	1	10.2±3	12.3±4.3	0.138	9.7±1.4	8.7±1	<0.0001	0.116

ŧ=measured in serum, þ=measured in plasmaContinuous data is presented as mean ± standard deviation, and categorical data is presented as percentage.A One-way ANOVA (normal data) or Kruskal Wallis test (non-normal data) was performed on the Δ values of each parameter to determine the difference between groups post intervention. Δ values were used to account for baseline differences. To determine the difference between baseline and post intervention, a paired t-test (normal data) or Wilcoxon signed rank test (non-normal data) was performed in SPSS. * used to indicate significance between the delta change of groups, as determined by a Bonferroni post hoc test

The impact of Leu ± LC n-3 PUFA supplementation on the proteomic composition of S-HDL particles over a 24-week period:

Within the current study we assessed the impact of Leu ± LC n-3 PUFA supplementation, on the proteomic composition of S-HDL and L-HDL particles in patients at risk of sarcopenia to determine whether strength-independent effects of dietary intervention were evident and captured within the HDL proteome.

The HDL proteome experienced distinct alterations in response to each supplement type (Figure 4 and Figure 5). The control supplement (containing mainly maltodextrin) caused a significant increase in *n*=14 proteins and significant decrease in *n*=11 proteins on S-HDL. The proteins that increased relative to baseline include TF ($p<0.05$), complement proteins complement C3 (C3) ($p<0.001$), complement C4A (C4A) ($p<0.05$) and C5 ($p<0.01$) and immunoglobulins (*n*=8). The proteins that decreased relative to baseline include apolipoprotein D (ApoD) ($p<0.05$) and apolipoprotein M (ApoM) ($p<0.05$), several inflammatory proteins including vitronectin (VTN) ($p<0.05$), GSN ($p<0.05$) and hemopexin (HPX) ($p<0.01$) and complement protein C6 (C6) ($p<0.05$) among others.

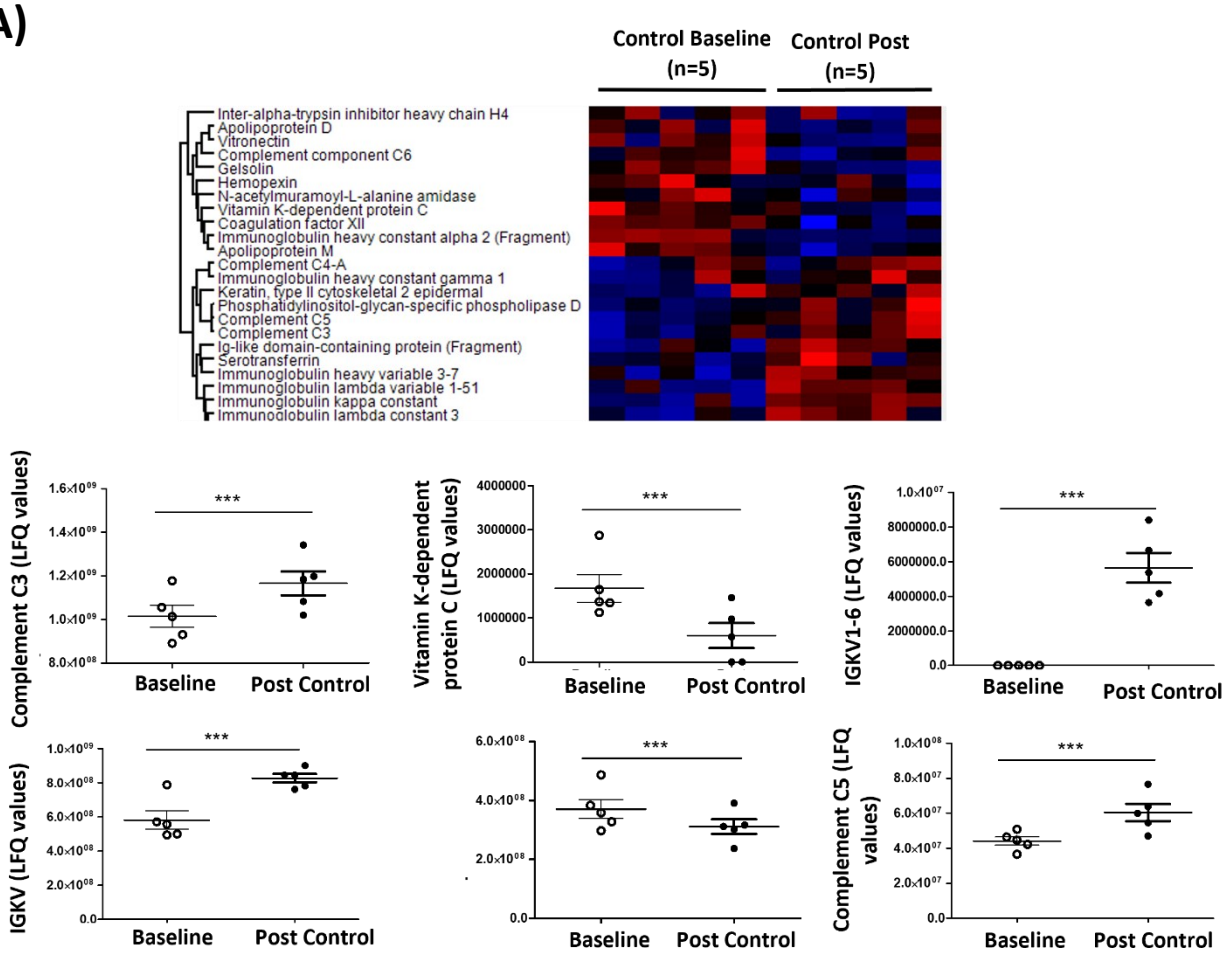
Leu supplementation significantly increased *n*=18 S-HDL proteins and significantly decreased *n*=9 proteins. The proteins that increased relative to baseline include immunoglobulins (*n*=16) and the complement proteins C3/C5 convertase ($p<0.01$) and C5 ($p<0.05$). The proteins that decreased relative to baseline include complement C2 (C2) ($p<0.01$), C1R ($p<0.05$), complement factor-H-related protein 1 (CFHR1) ($p<0.05$), apolipoprotein A-II (ApoA2) ($p<0.05$) and alpha-2-antiplasmin (SERPINF2) ($p<0.05$) (Figure 4B).

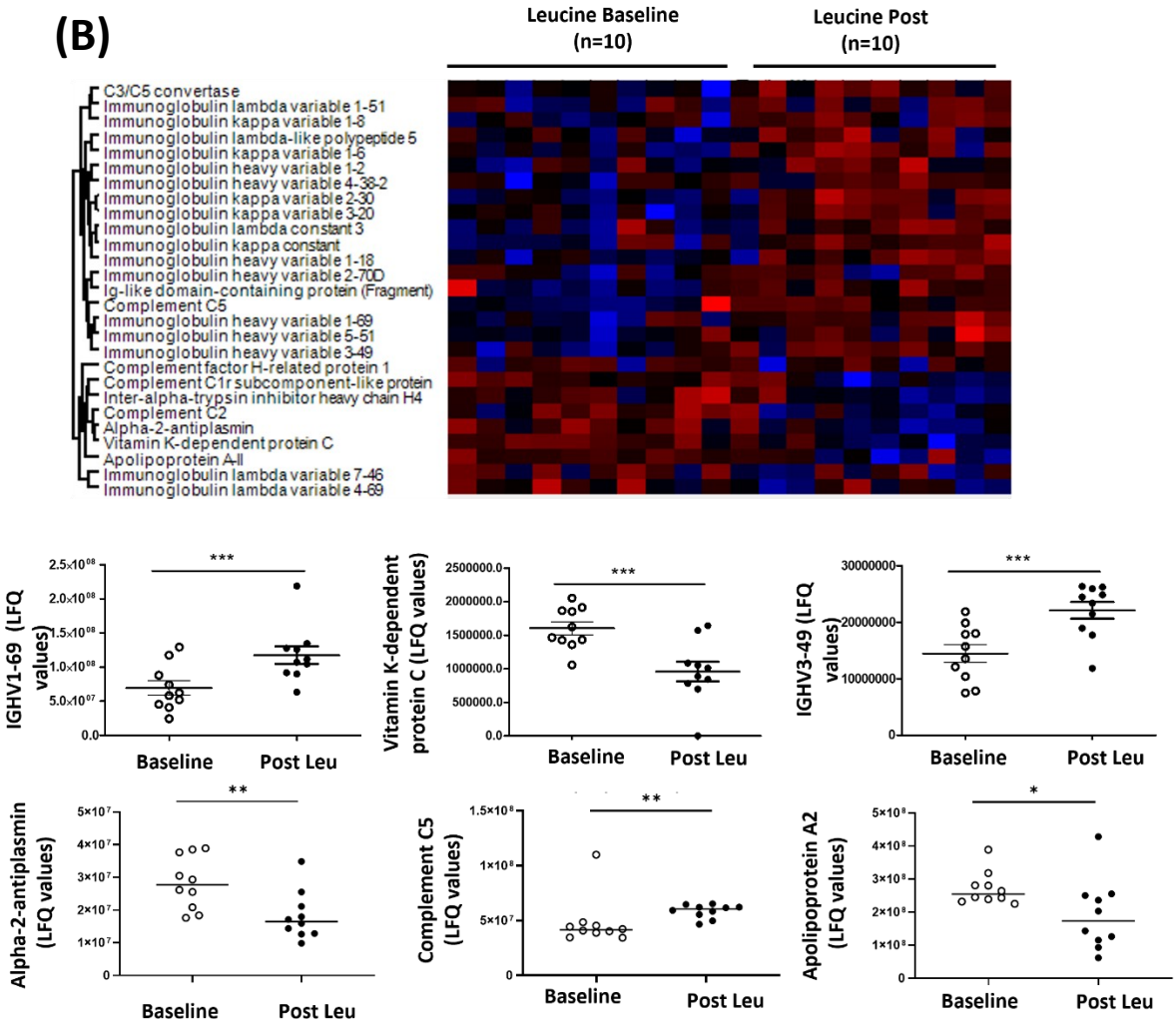
The combination of Leu + LC n-3 PUFA significantly increased *n*=8 proteins and significantly decreased *n*=10 proteins. The proteins that significantly increased relative to baseline include immunoglobulins (*n*=7) and C5 ($p<0.001$). The proteins that significantly decreased relative to baseline include the inflammation related proteins GSN ($p<0.01$), alpha-2-HS-glycoproteins (AHSG) ($p<0.01$) and alpha-1-antitrypsin (SERPINA1) ($p<0.05$), apolipoprotein A1 (ApoA1) ($p<0.05$) and complement factor-H-related protein 2 (CFHR2) ($p<0.05$).

There was a notable overlap in the effects of Leu alone and Leu + LC n-3 PUFA supplementation on the proteomic composition of S-HDL particles with *n*=6 proteins significantly modulated by both interventions, including several immunoglobulins (*n*=5) and SERPINF2 (Figure 5). A number of immunoglobulins (*n*=4) were significantly modulated in both the control and Leu groups, but not the Leu ± LC n-3 PUFA group. One protein GSN was significantly modulated in both the control ($p<0.05$)

and Leu + n-3 PUFA group ($p<0.01$), but not the Leu alone group. A total of *n*=4 proteins were significantly modulated across all dietary intervention groups including Vitamin K-dependent protein C (PROC), IGLC3, Inter-alpha-trypsin inhibitor heavy chain H4 (ITIH4), and C5. All the raw data of the significant proteins is displayed in supplementary table 1.

(A)


Control Baseline (n=5)
Control Post (n=5)
Inter-alpha-trypsin inhibitor heavy chain H4
Apolipoprotein D
Vitronectin
Complement component C6
Gelsolin
Hemopexin
N-acetylmuramoyl-L-alanine amidase
Vitamin K-dependent protein C
Coagulation factor XII
Immunoglobulin heavy constant alpha 2 (Fragment)
Apolipoprotein M
Complement C4-A
Immunoglobulin heavy constant gamma 1
Keratin, type II cytoskeletal 2 epidermal
Phosphatidylinositol-glycan-specific phospholipase D
Complement C5
Complement C3
Ig-like domain-containing protein (Fragment)
Serotransferrin
Immunoglobulin heavy variable 3-7
Immunoglobulin lambda variable 1-51
Immunoglobulin kappa constant
Immunoglobulin lambda constant 3
Complement C3 (LFQ values)
Vitamin K-dependent protein C (LFQ values)
IGKV1-6 (LFQ values)
IGKV (LFQ values)
Complement C5 (LFQ values)
***
Baseline
Post Control


(B)
Leucine Baseline (n=10)
Leucine Post (n=10)
C3/C5 convertase
Immunoglobulin lambda variable 1-51
Immunoglobulin kappa variable 1-8
Immunoglobulin lambda-like polypeptide 5
Immunoglobulin kappa variable 1-6
Immunoglobulin heavy variable 1-2
Immunoglobulin heavy variable 4-38-2
Immunoglobulin kappa variable 2-30
Immunoglobulin kappa variable 3-20
Immunoglobulin lambda constant 3
Immunoglobulin kappa constant
Immunoglobulin heavy variable 1-18
Immunoglobulin heavy variable 2-70D
Ig-like domain-containing protein (Fragment)
Complement C5
Immunoglobulin heavy variable 1-69
Immunoglobulin heavy variable 5-51
Immunoglobulin heavy variable 3-49
Complement factor H-related protein 1
Complement C1r subcomponent-like protein
Inter-alpha-trypsin inhibitor heavy chain H4
Complement C2
Alpha-2-antiplasmin
Vitamin K-dependent protein C
Apolipoprotein A-II
Immunoglobulin lambda variable 7-46
Immunoglobulin lambda variable 4-69
IGHV1-69 (LFQ values)
Vitamin K-dependent protein C (LFQ values)
IGHV3-49 (LFQ values)
Alpha-2-antiplasmin (LFQ values)
Complement C5 (LFQ values)
Apolipoprotein A2 (LFQ values)
Baseline
Post Leu
***
**
*

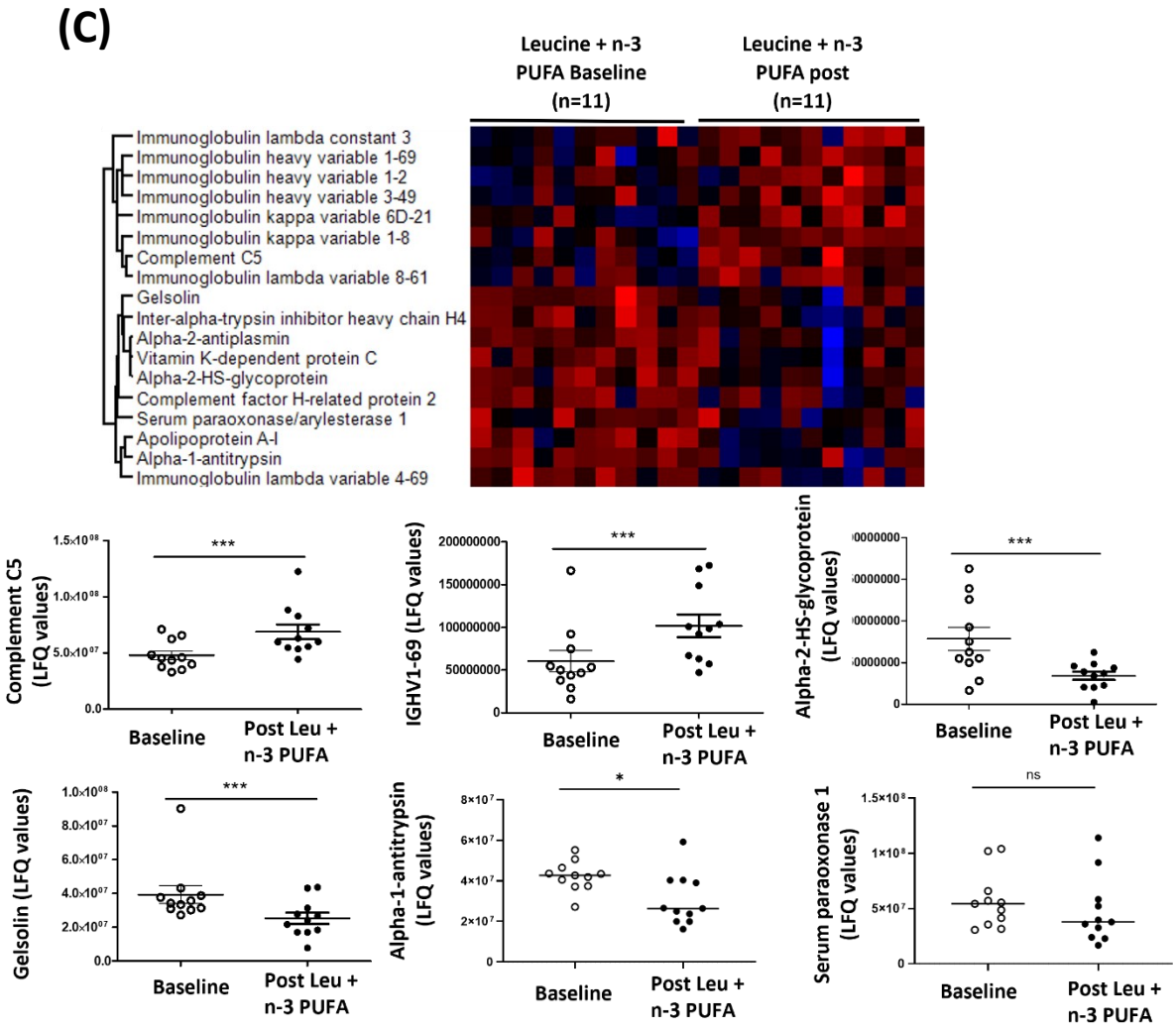


Figure 4: Heatmaps and dot plots representing the proteins that are significantly modulated on S-HDL in (A) control, (B) Leu alone and (C) Leu + fish-oil groups. Dot plots show the LFQ values of the top 6 most significant proteins as determined by t-test in Perseus. Significance of LFQ values was determined in GraphPad for display purposes. p***≤0.001,p** ≤0.01,p*≤0.05

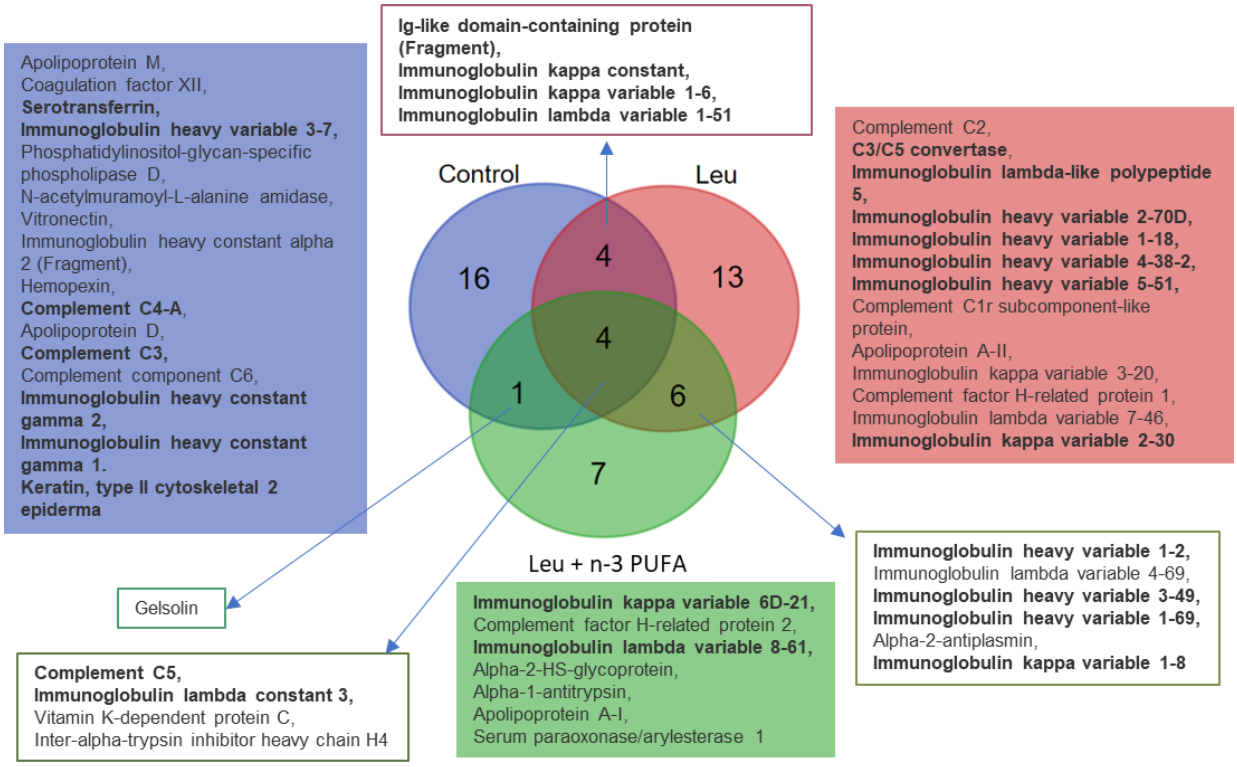


Figure 5: Venn diagram showing the significant proteins unique and shared between each dietary intervention group on S-HDL particles. Bolded proteins are upregulated proteins.

The impact of Leu ± LC n-3 PUFA supplementation on the proteomic composition of L-HDL particles over a 24-week period:

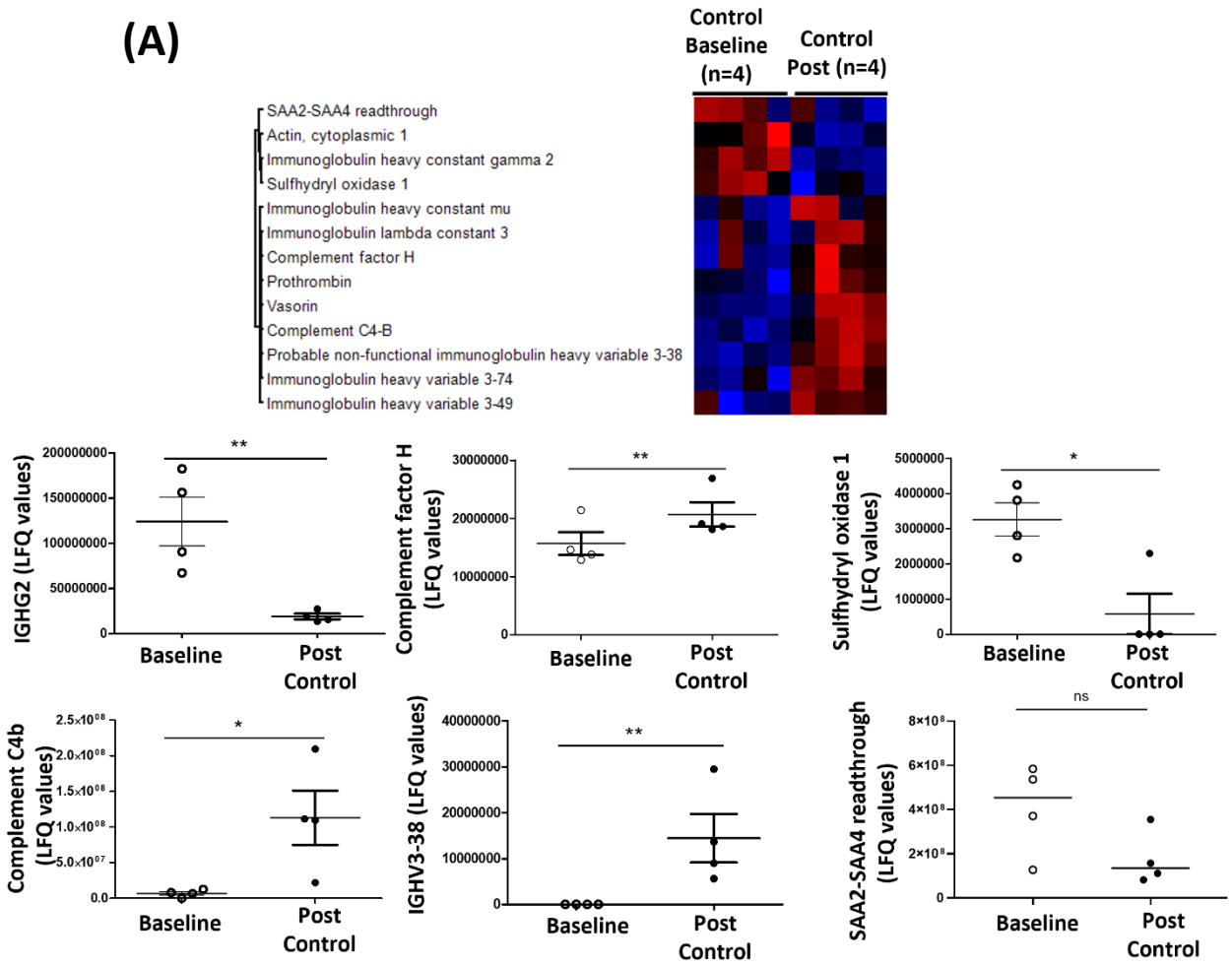
Changes in the proteomic composition of L-HDL particles in response to Leu ± n-3 PUFA or maltodextrin control were subsequently investigated and again the composition of the particles was uniquely affected by each supplement type (Figure 6 and Figure 7).

The control supplement caused a significant increase in *n*=9 proteins and a significant reduction in *n*=4 proteins on L-HDL. The proteins that significantly increased relative to baseline include immunoglobulins (*n*=5), C4B ($p<0.05$), Vasorin (VSN) ($p<0.05$) and F2 ($p<0.05$). The proteins that significantly decreased include QSOX1 ($p<0.05$), SAA2-SAA4 readthrough (SAA2-SAA4) ($p<0.05$), actin cytoplasmic 1 (ATCB) ($p<0.05$), and IGHG2 ($p<0.05$) (Figure 6A).

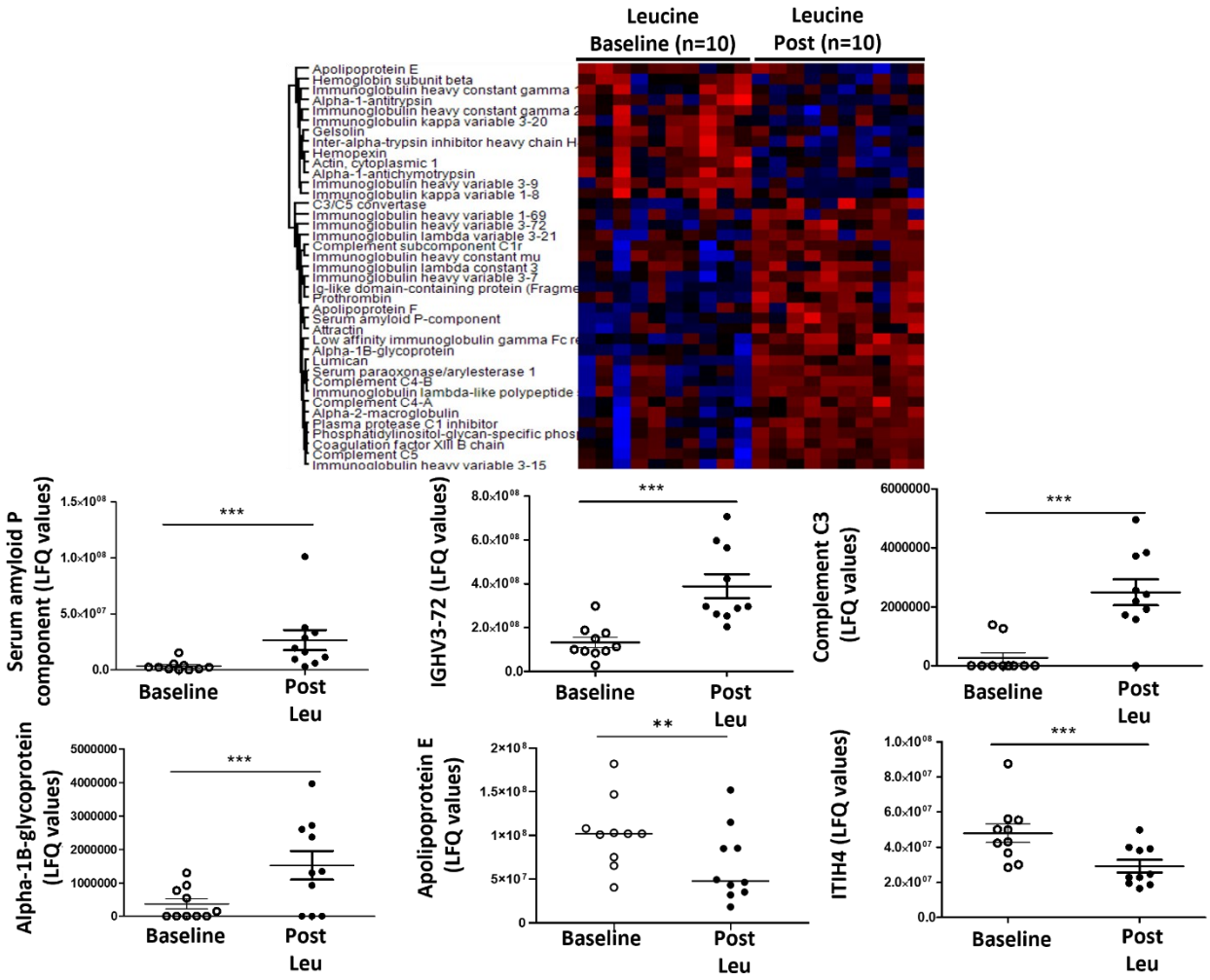
The Leu supplement caused a significant increase in *n*=23 proteins and a significant decrease in *n*=13 proteins on L-HDL. The proteins that significantly increased relative to baseline include immunoglobulins (*n*=10) and the complement proteins, C3/C5 convertase ($p<0.05$), C1R ($p<0.05$), C4B ($p<0.01$), C4A ($p<0.01$) and C5 ($p<0.05$). The proteins that significantly decreased include alpha-2-macroglobulin (A2M) ($p<0.05$), alpha-1B-glycoprotein (A1BG) ($p<0.001$), PON1 ($p<0.01$) and ApoF ($p<0.01$) (Figure 6B).

The Leu + LC n-3 PUFA supplement resulted in a significant increase in *n*=20 proteins and a significant decrease in *n*=19 proteins on L-HDL. The proteins that increased relative to baseline include immunoglobulins (*n*=9), plasma kallikrein ($p<0.01$), A1BG ($p<0.05$), serum amyloid-P component (APCS) ($p<0.05$), F2 ($p<0.05$) and C4B ($p<0.05$). The proteins that were significantly decreased post Leu + n-3 PUFA supplementation include the apolipoproteins ApoA1 ($p<0.01$), ApoA2 ($p<0.01$), Apolipoprotein A-IV (ApoA4) ($p<0.01$), Apolipoprotein B (ApoB) ($p<0.05$), Apolipoprotein C1 (ApoC1) ($p<0.0001$), ApoD ($p<0.001$), ApoE ($p<0.05$) and Apolipoprotein L1 (ApoL1) ($p<0.05$). Other proteins which decreased relative to baseline include C7 ($p<0.001$), SAA2-SAA4 ($p<0.001$) and SELENOP ($p<0.05$) (Figure 6C).

The greatest similarity between groups was observed between Leu and Leu + n-3 PUFA, with *n*=9 proteins shared between these groups including immunoglobulins (*n*=5), Lumican (LUM) ($p<0.05$ for both), APCS ($p<0.0001$ and $p<0.001$ respectively), A1BG ($p<0.001$ and $p<0.05$ respectively) and ApoE ($p<0.01$ and $p<0.05$ respectively) (Figure 7). A further *n*=3 proteins including C4B, F2, and ATCB were significantly modulated on L-HDL particles in response to all supplement types. All the raw data of the significant proteins is displayed in supplementary table 1.


(A)
Control Baseline (n=4)
Control Post (n=4)
SAA2-SAA4 readthrough
Actin, cytoplasmic 1
Immunoglobulin heavy constant gamma 2
Sulfhydryl oxidase 1
Immunoglobulin heavy constant mu
Immunoglobulin lambda constant 3
Complement factor H
Prothrombin
Vasorin
Complement C4-B
Probable non-functional immunoglobulin heavy variable 3-38
Immunoglobulin heavy variable 3-74
Immunoglobulin heavy variable 3-49
IGHG2 (LFQ values)
Complement factor H (LFQ values)
Sulfhydryl oxidase 1 (LFQ values)
Complement C4b (LFQ values)
IGHV3-38 (LFQ values)
SAA2-SAA4 readthrough (LFQ values)
Baseline
Post Control
**
*
ns

(B)


Leucine
Baseline (n=10)
Leucine
Post (n=10)
Apolipoprotein E
Hemoglobin subunit beta
Immunoglobulin heavy constant gamma 1
Alpha-1-antitrypsin
Immunoglobulin heavy constant gamma 2
Immunoglobulin kappa variable 3-20
Gelsolin
Inter-alpha-trypsin inhibitor heavy chain H
Hemopexin
Actin, cytoplasmic 1
Alpha-1-antichymotrypsin
Immunoglobulin heavy variable 3-9
Immunoglobulin kappa variable 1-8
C3/C5 convertase
Immunoglobulin heavy variable 1-69
Immunoglobulin heavy variable 3-72
Immunoglobulin lambda variable 3-21
Complement subcomponent C1r
Immunoglobulin heavy constant mu
Immunoglobulin lambda constant 3
Immunoglobulin heavy variable 3-7
Ig-like domain-containing protein (Fragme
Prothrombin
Apolipoprotein F
Serum amyloid P-component
Attractin
Low affinity immunoglobulin gamma Fc re
Alpha-1B-glycoprotein
Lumican
Serum paraoxonase/arylesterase 1
Complement C4-B
Immunoglobulin lambda-like polypeptide
Complement C4-A
Alpha-2-macroglobulin
Plasma protease C1 inhibitor
Phosphatidylinositol-glycan-specific phos
Coagulation factor XIII B chain
Complement C5
Immunoglobulin heavy variable 3-15
Serum amyloid P component (LFQ values)
1.5x10^08
1.0x10^08
5.0x10^07
0.0
***
Baseline
Post Leu
IGHV3-72 (LFQ values)
8.0x10^08
6.0x10^08
4.0x10^08
2.0x10^08
0.0
***
Baseline
Post Leu
Complement C3 (LFQ values)
6000000
4000000
2000000
0
***
Baseline
Post Leu
Alpha-1B-glycoprotein (LFQ values)
5000000
4000000
3000000
2000000
1000000
0
***
Baseline
Post Leu
Apolipoprotein E (LFQ values)
2x10^8
1.5x10^8
1x10^8
5x10^7
0
**
Baseline
Post Leu
ITIH4 (LFQ values)
1.0x10^08
8.0x10^07
6.0x10^07
4.0x10^07
2.0x10^07
0.0
***
Baseline
Post Leu

(C)

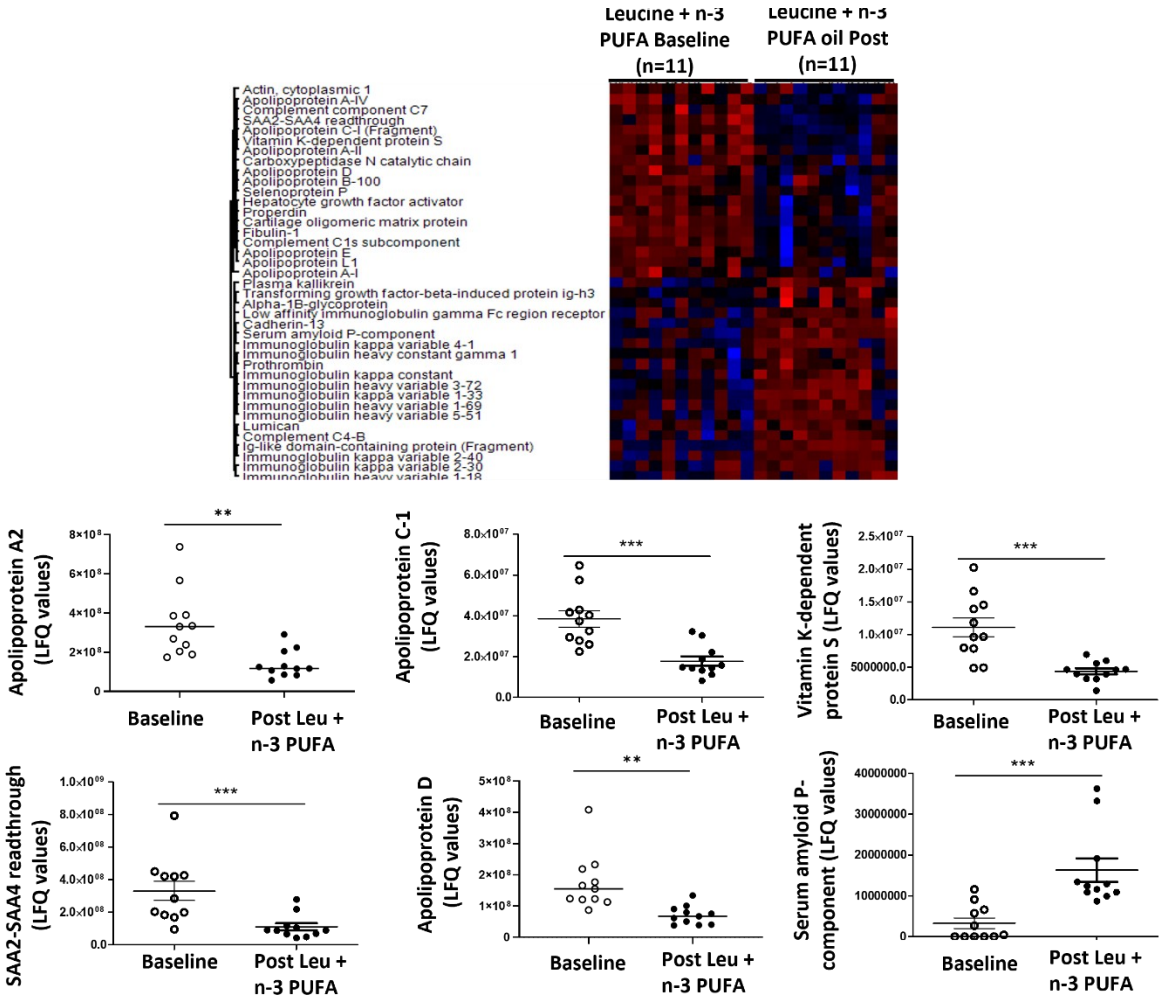


Figure 6: Heatmaps and dot plots representing the proteins that are significantly modulated on S-HDL in (A) control, (B) Leu alone and (C) Leu + fish-oil groups. Dot plots show the LFQ values of the top 6 most significant proteins as determined by t-test in Perseus. Significance of LFQ values was determined in GraphPad for display purposes. p***≤0.001,p** ≤0.01,p*≤0.05

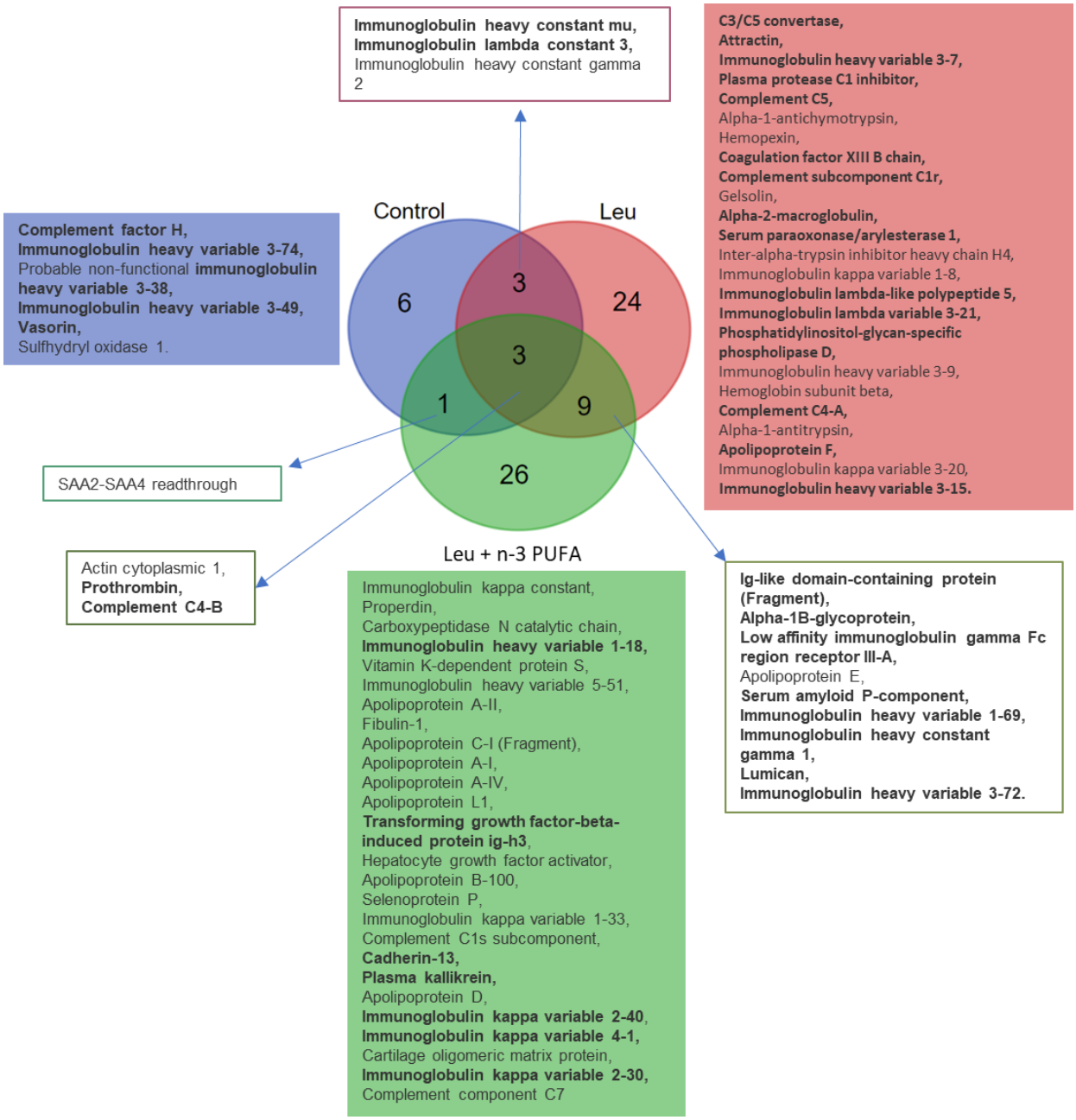


Figure 7: Venn diagram showing the significant proteins unique and shared between each dietary intervention group on L-HDL particles. Bolded proteins are upregulated proteins.

The relationship between changes in HDL-associated proteins from baseline and changes in measures of strength/SMI over the intervention

To examine the dynamic relationship between alterations in strength/SMI and changes in HDL composition over time, we correlated the delta change (Δ) of S-HDL and L-HDL-associated proteins across all groups combined with the delta change (Δ) of handgrip-strength, Δ leg-strength and Δ SMI (Table 6) pre-versus post-intervention.

Changes in handgrip-strength positively correlated with *n*=5 proteins and negatively correlated with *n*=4 Δ proteins on S-HDL. The proteins Δ F2 (r=0.579, p=0.0024), Δ AHSG (r=0.506, p=0.0099), Δ coagulation factor XII (F12) (r=0.506, 0.0229), Δ ApoD (r=0.427, p=0.0333) and Δ ceruloplasmin (CP) (r=0.4, p=0.0477) positively correlated with Δ handgrip-strength while immunoglobulin lambda variable 3-21 (IGLV3-21) (r=-0.589, p=0.0063), IGKV1-17 (r=-0.594, p=0.0119), immunoglobulin heavy variable 3-7 (IGHV3-7) (r=-0.529, p=0.0065) and C3 (r=-0.472, p=0.0173) negatively correlated with Δ handgrip-strength.

Δ leg-strength positively correlated with *n*=1 proteins on S-HDL, heparin cofactor 2 (SERPIND1) (r=0.437, p=0.0255), and negatively correlated with *n*=4, Δ immunoglobulin lambda variable 3-25 (IGLV3-25) (r=-0.578, p=0.0385), Δ immunoglobulin lambda variable 1-47 (IGLV1-47) (r=-0.542, p=0.0092), Δ QSOXI (r=-0.517, p=0.0255), Δ immunoglobulin lambda constant 7 (IGLC7) (r=-0.541, p=0.0167). Δ SMI correlated with changes in only *n*=1 protein on S-HDL, Δ coagulation factor IX (F9) (r=0.523, p=0.0125).

There were no significant associations between Δ L-HDL proteins with Δ handgrip-strength. Δ leg-strength positively correlated with changes in *n*=1 proteins, Δ C3/C5 convertase (r=0.486, p=0.0138), and negatively correlated with changes in *n*=3 proteins, Δ A2M (-0.572, p=0.0035), Δ Vitamin K-dependent protein S (PROS1) (r=-0.427, p=0.042) and Δ IGLC3 (r=-0.421, p=0.036) on L-HDL particles.

Δ SMI correlated with only *n*=1 protein on L-HDL, IGLC3 (r=-0.439, 0.0411). Δ IGLC3 correlated with Δ SMI, Δ leg-strength change % and Δ leg-strength, all of which were negatively associated with this protein.

Table 6: Correlation table of Δ handgrip-strength, Δ leg-strength and ΔSMI with Δ S- and L-HDL associated proteins across all participants combined

	Δ S-HDL			Δ L-HDL		
	Δ handgrip -strength (Kg)	**Δ leg-strength (MVC nM)**	**Δ Skeletal muscle index (kg/m²)**	**Δ handgrip -strength (Kg)**	**Δ leg-strength (MVC nM)**	**Δ Skeletal muscle index (kg/m²)**
F2	0.579**	0.149	-0.333	0.03	0.077	0.183
IGLV3-21	-0.589**	-0.026	-0.073	N/A	N/A	N/A
IGHV3-7	-0.529**	0.364	-0.132	0.266	-0.047	-0.354
AHSG	0.506**	-0.227	-0.147	N/A	N/A	N/A
IGKV1-17	-0.594*	-0.251	0.227	N/A	N/A	N/A
C3	-0.472*	0.285	-0.09	0.06	-0.125	0.071
F12	0.506*	0	-0.225	N/A	N/A	N/A
APOD	0.427*	-0.117	-0.23	0.218	-0.058	-0.154
CP	0.4*	0.132	-0.059	0.033	0.209	0.212
F9	0.045	0.016	0.523*	N/A	N/A	N/A
SERPIND1	0.049	0.437*	0.033	0.376	0.296	0.027
IGLV3-25	0.022	-0.578*	0.204	N/A	N/A	N/A
IGLV1-47	0.063	-0.542**	-0.188	N/A	N/A	N/A
QSOX1	-0.152	-0.517*	0.021	N/A	N/A	N/A
IGLC7	-0.283	-0.541*	0	N/A	N/A	N/A
IGKV2D-28	-0.077	-0.467	-0.34	N/A	N/A	N/A
A2M	N/A	N/A	N/A	0.188	-0.572**	0.051
ALB	-0.117	-0.035	-0.159	-0.139	0.498*	-0.188
PROS1	0.317	-0.078	-0.357	0.168	-0.427*	-0.127
IGLC3	-0.288	0.059	0.12	0.11	-0.421*	-0.439*
C3/C5	-0.089	0.311	0.043	0.143	0.486*	-0.033

Values displayed are correlation coefficients (r). Correlations were carried out in GraphPad Prism version 10. Pearson correlation was used for normally distributed data while Spearman correlation was used for non-normally distributed data. p-values displayed as *. p*<0.05, p**<0.01, p***<0.001. Significant cells are highlighted based on the strength of the correlation. Darker red indicates a stronger positive correlation and darker blues indicate a stronger negative correlation. NA is displayed for proteins that did not have sufficient data points at baseline or post supplementation to calculate a Δ value or were not present on S- or L-HDL.

Discussion

This study has shown for the first time that the HDL proteome exhibits unique signatures that can potentially differentiate between patients at lower and higher risk of sarcopenia in adults aged over 65 years. These markers hold promise for risk assessment, aiding in the identification of those most in need of intervention. Our study also demonstrates for the first time, the sensitivity of measuring HDL-associated proteins at tracking responses to dietary interventions that move beyond traditional biochemical biomarkers. The HDL proteome provided considerable insights into both the metabolic state (particularly apolipoprotein metabolism) and inflammatory state in individuals pre- and post-dietary intervention and could capture unique differences in response to leucine supplementation alone versus leucine + LC n-3 PUFA supplementation. The HDL proteome therefore represents a potentially novel immune-metabolic biomarker to aid precision nutrition studies, particularly in those with features of metabolic dysfunction including sarcopenia.

Biomarkers to identify patients at high risk of sarcopenia represent a major unmet need. Within this study we identified significant increases in prothrombin (F2), complement C4-B, ceruloplasmin (CP) and serum paraoxonase-1 (PON-1), and decreased association of a number of immunoglobulins (IGs) on S-HDL particles in individuals at high-risk of sarcopenia relative to lower-risk individuals. CP, the main copper carrying protein in circulation and an acute phase protein[307], is also increased on HDL during inflammation where it impairs HDL's ability to inhibit LDL oxidation[227] and negatively associates with cholesterol efflux capacity[298]. Elevated serum CP levels are indicative of increased oxidative stress[308] and have been found to be a marker of disease in type-II diabetes[308], metabolic syndrome[307], and coronary heart disease[309] and thus enrichment of HDL with CP may be an important biomarker of low-grade inflammation, a causal contributor to sarcopenia[274].

Presence of IGs on HDL has been debated and have historically been dismissed as contaminants of the HDL isolation process[310]. However they are repeatedly identified in HDL proteome studies[311] despite different isolation techniques [312],[313], [314]. Within the current study we applied an additional enrichment step to further purify our FPLC fractions for only lipid containing vesicles (LRA pull-down step) and thus our studies suggest that the IGs are indeed present within the HDL fractions. The functional role for IGs on HDL is however unknown, and HDL may just serve as a vehicle to clear these protein fragments from circulation.

L-HDL particles carry a distinct protein cargo relative to S-HDL particles with particular enrichment of IGs on S-HDL and apolipoproteins on L-HDL particles[306, 315] . We therefore sought to analyse the proteomic composition of S-HDL and L-HDL particles separately. As anticipated, a different set of

proteins were modulated between patients at moderate-risk versus high-risk of sarcopenia on L-HDL particles relative to S-HDL. QSOX1, prothrombin (F2) and vitronectin (VTN) were reduced on L-HDL of the high-risk group while actin cytoplasmic 1 (ACTB) and albumin were increased. On S-HDL of the high-risk group CP, C4B, and PON1 were among the proteins increased while several IGs were decreased. QSOX1 is an endoplasmic reticulum, Golgi apparatus and secretory enzyme that catalyses disulphide bonds, ensuring correct protein folding[316]. It is involved in extracellular matrix integrity[317], prevents apoptosis in response to oxidative stress[318] and inhibits autophagy in response to amino acid starvation[319]. PON1 is a HDL associated protein with anti-inflammatory and anti-oxidative effects. It is particularly associated with cardiovascular disease and somewhat contradictory to our results, lower PON1 activity was associated with age and inflammation in an elderly population[320].

The HDL proteome carries up to 17 of the 30 complement proteins, which represents an intrinsic part of the innate immune system[321, 322], including the inhibitors clusterin (ApoJ) and VTN[322, 323] and participating proteins C3, C4 and C5[303, 311]. Complement proteins have previously been reported to be enriched on HDL during inflammatory conditions such as rheumatoid arthritis[324] and cardiovascular disease[303], while the complement system has been implicated in aging[325] and sarcopenia[326]. Many of the complement proteins (eg C4B, C5 and C6) identified in this study negatively correlated with handgrip or leg-strength but did not show a significant relationship with SMI. The typically negative association between complement proteins and strength suggest that increased complement activation coincides with strength loss. Additionally, C4-B was upregulated on S-HDL in participants at high-risk of sarcopenia while VTN, a negative regulator of complement[327], was down regulated on L-HDL. C4B plays a role in the classical complement pathway and while not much is known about its role in ageing, it was found to increase across the lifespan in the gastrocnemius, liver and kidney of rats[328]. Additionally, in a 3D model of aged muscle, an increase in C4-B impaired the regenerative capacity of muscle cells, which was improved by C4B inhibition[329]. VTN, in addition to playing a role in complement activation[327], is an acute phase protein[330], regulates coagulation and fibrinolysis[331] and is involved in cell adhesion and migration[332]. Similar to our study, a number of complement related proteins (C5,C6,C7,C9,C1s etc) were upregulated in serum of frail patients >65 years of age (*n*=12)[333]. Many of these biomarkers of frailty correlated with strength/muscle mass in our cohort including C7, VTN, C6, C5, and C1S[333], supporting the role of complement in strength loss. Notably changes in C3 on S-HDL (ΔC3) negatively correlated with Δ handgrip-strength (r=-0.472) over the course of the dietary intervention. A previous cross-sectional study found that serum C3 levels were lower in older individuals (*n*=269) with low muscle mass (pre-sarcopenia), low handgrip-strength (dynapenia) and sarcopenia, compared to robust individuals[326] which is conflicting with our findings. However, C3 is also an acute phase protein[334], and was upregulated in the plasma of mice with cancer cachexia[335]

while C3a was upregulated in the serum of inflamed cancer patients with cachexia, highlighting its role in muscle wasting[336]. A major difference between our study and others is we specifically measured HDL-associated C3 while previous studies have measured C3 in serum specifically.

A major challenge with sarcopenia is the number of different metrics that can be utilized to assess strength. Within this study we demonstrate that while the different metrics of strength (handgrip strength, leg-strength and SMI) all significantly correlate with each other at baseline, there were very distinct associations between HDL-proteins and each specific measure of strength. For instance, we demonstrated strong associations between S-HDL-associated ApoD and handgrip strength (r=0.435) and leg strength (r=0.581) but no association to SMI (r=0.091). ApoD is a lipocalin protein that is expressed in many tissue types but is primarily bound to HDL in circulation[337]. It plays a role in lipid metabolism[337], oxidative stress[338] and nerve repair[339] and has been identified as an acute phase protein[338]. While elevated levels are thought to be protective against oxidative stress[340], levels increase in age-induced muscle atrophy[341] and aging[342-344]. Notably, serum ApoD, along with several other apolipoproteins, was downregulated in a group of healthy men (*n*=20) in response to 60 days of bed rest[345] while ApoD has also been found to be associated with increased longevity and stress resistance in mice[346] and drosophila melanogaster[347]. It is possible the positive association of ApoD and strength we see in our study is indicative of a robust response against oxidative stress, a known contributor to strength loss[348] and sarcopenia[349]. Both complement factors 5 (C5) (r=-0.398) and 6 (C6) (r=-0.403) negatively associated with handgrip strength but not leg-strength or SMI at baseline. By contrast, a number of IGs including IGHV1-18 (r=0.575) positively correlated with SMI, but not with handgrip strength or leg strength. HDL-associated SERPING1 correlated most strongly with SMI (r=-0.741) and handgrip strength (r=-0.628) but did not reach significance for leg strength (r=-0.346). SERPING1 helps to regulate the complement cascade by inhibiting components of the C1 complex, and also inhibits activation of plasminogen and the kallikrein–kinin system, responsible for vascular permeability[350, 351]. Consistent with more abundant amounts of apolipoproteins on L-HDL particles, we saw significant (and negative) associations between a number of apolipoproteins (ApoA1, ApoE and ApoM) and measures of strength. We also observed a significant negative association between the liver-derived acute phase proteins[352], SAA2-SAA4 on L-HDL and measures of leg strength (r=-0.474) and SMI (r=-0.472) but not handgrip strength (r=-0.335). The majority of SAA in circulation is bound to HDL[353]. Increases in this protein creates dysfunctional pro-inflammatory HDL[354, 355] by displacing ApoA1[227, 356], preventing HDL interacting with the plasma membrane[355] and inducing the expression of monocyte chemoattractant protein-1 (MCP1)[354, 357]. Conversely, the accumulation of SAA on HDL has been hypothesised to neutralise its pro-inflammatory effects[353] and even protect HDL from oxidation[358, 359]. SAA reportedly increases in circulation[360, 361] and on HDL[362] with age. It has also been

implicated (mainly subtype SAA1) in inflammation-mediated muscle wasting in patients with critical illness myopathy (*n*=30)[363] and cancer cachexia (*n*=35)[335] as well as in in mouse models of cachexia[335], persistent inflammation, immunosuppression, and catabolism syndrome (PICS)[364] and angiotensin II induced muscle wasting[365]. The increase in SAA2-SAA4 on HDL in individuals with the lowest SMI and leg-strength in our study likely reflects an increased inflammatory state, which is a known contributor to sarcopenia[274]. The protein gelsolin (GSN) was the only protein on HDL that significantly correlated with all measurements of strength (leg-strength (r=0.551), handgrip-strength (r=0.504) and SMI (r=0.429)). Plasma GSN is mainly produced by muscle[366] and is known to play a role in cytoskeleton remodelling[367], neutralisation of inflammatory stimulants such as actin filaments[368], LPS[369] and platelet-activating factor (PAF)[370], and oxidative stress[371]. High plasma levels of GSN have been identified as a biomarker of robust ageing and reduced mortality in a group of community dwelling men *n*=469 (~83 years old)[372]. Interestingly, GSN is positively correlated with actin, cytoplasmic 1 (ACTB) on both S- and L- baseline HDL (Supplementary figure 1) suggesting increased GSN expression may be a protective mechanism against increased circulating actin filaments, an indicator of tissue injury[373]. Furthermore, ACTB was upregulated on L-HDL in those at high-risk of sarcopenia.

Notably, we did not observe significant associations within IGs on L-HDL and SMI, as was observed with S-HDL particles, which likely reflects the unique protein cargos of S- and L-HDL particles respectively. The functional consequences of differences in protein composition between L- and S-HDL particles are not fully understood and to date most studies measuring the HDL proteome pool all HDL particles for analysis[312, 374-376]. The current study highlights the importance of analysing these lipoprotein sub-fractions separately, particularly for biomarker discovery.

Dietary and/or lifestyle intervention represents an important prophylactic approach to prevent, delay or reverse significant muscle wastage in high-risk individuals[377, 378]. However, to date diagnosis of sarcopenia is only possible after significant muscle wasting making preventative approaches difficult to design and demonstrate efficacy thereof. We therefore sought to investigate the impact of dietary intervention with the anabolic protein leucine with and without anti-inflammatory LC n-3 PUFA within the Nutrimal study on biomarkers of metabolism and inflammation (that are captured within the HDL proteome), to elucidate whether there were strength independent effects of dietary intervention in patients at high-risk of sarcopenia[289]. The findings from our study demonstrate potent and unique changes within the HDL proteome in response to all the dietary interventions (maltodextrin control, leucine alone and leucine combined with LC n-3 PUFA). Leu intervention increased levels of IGs and C5, and reduced levels of ApoA2, complement C2 and SERPINF2 on S-HDL particles, while the combination of Leu + LC n-3 PUFA significantly increased levels of IGs and C5, and reduced GSN, alpha-2-HS glycoproteins, Complement components (C7 and C1s), SERPINA1 and ApoA1 on S-HDL particles.

Downregulation of complement proteins in response to n-3 PUFA was previously shown in a study by Burillo et al[379] wherein n-3 PUFA supplementation (2g daily for 5 weeks) in male smokers (*n*=6) downregulated C1r and CF levels[379]. Conversely, increased complement activation (including C3,C4b, and complement factor B) was found in the liver and serum of mice fed a diet high in fish oil compared to a low fat diet[380]. Down-regulation of GSN in response to the different dietary interventions, which is positively associated with all measures of muscle strength, would be projected to be potentially detrimental for patients at high-risk of sarcopenia. However, it is noteworthy that we found no association between changes in GSN upon dietary intervention and changes in muscle strength over the course of the study. Yang et al., previously showed that EPA significantly reduced HDL associated GSN while DHA had no effect in a small intervention cohort (*n*=10)[381] which was consistent with our findings. It is notable in our study that the caloric control supplement (containing maltodextrin) had its own distinct effects on the HDL proteome, albeit to a lesser extent than either the Leu ± LC n-3 PUFA arms. Most notably we observed reductions in ApoD, VTN, GSN and ApoM, and enrichment with C3, C5 and immunoglobulins on S-HDL and depletion of SAA2-4 and enrichment of C4-B on L-HDL. To date only one study, by Andraski et al., has looked at the effects of carbohydrates on the HDL proteome[382]. They found that replacing monounsaturated fats with carbohydrates increased the fractional catabolic rate of ApoM on L-HDL but not the production rate of the protein. This suggests an increased demand for ApoM in a high carbohydrate diet or increased clearance of HDL containing ApoM[382] which may contribute to the decrease we see in our study.

The metabolic effects of LC n-3 PUFA were particularly evident within the proteomic composition of L-HDL particles with downregulation of multiple LXR-induced apolipoproteins evident (ApoA1, ApoA2, ApoA4, ApoB, ApoC1, ApoD, ApoE and ApoL1). These effect of LC n-3 PUFA on apolipoprotein levels are likely coupled to the reduction in plasma triglycerides (p=0.051) evident within this intervention group. Increased beta oxidation/PPAR alpha activation in response to LC n-3 PUFA is widely reported [383, 384] and is in turn associated with reductions in LXR activation in the liver[385, 386]. Grytten et al. previously demonstrated that n-3 PUFA reduced ApoAII, but not ApoAI, in a cohort of men and women with abdominal obesity (*n*=39) [387]. The effects of n-3 PUFA on ApoA1 are variable with increased[379], reduced[388] and unchanged[389, 390] levels reported. This variability could be due to differences in dose, duration as well as the population used. ApoL1 was also depleted from L-HDL in the Leu + n-3 PUFA group within our study, consistent with findings from de Roos *et al* wherein serum ApoL1 decreased in healthy adults (*n*=81) after LC n-3 PUFA supplementation, compared to n-6 PUFA[388]. ApoE on L-HDL was downregulated in both the Leu and Leu + LC n-3 PUFA arms of the Nutrimal intervention suggesting that this effect is attributable to the leucine component. ApoE plays a role in many steps of the RCT system from HDL biosynthesis to hepatic cholesterol uptake[391] while also facilitating the rapid

clearance of HDL via the liver after a high-fat diet[391]. By contrast ApoE enrichment on HDL_3 particles (small/medium HDL[392]) was identified by Vaisar et al., as a biomarker of CAD (*n*=77)[303]. An increase in HDL associated ApoE was also found in patients with severe liver cirrhosis compared to cases of mild cirrhosis and healthy controls (*n*=100)[393]. Another study (*n*=1112) found that ApoE enriched HDL were produced in response to particles becoming "overloaded" with cholesterol, a feature associated with higher incidence of coronary heart disease[394]. However, they determined that this increase in ApoE mitigated the effects of overladen HDL particles and attenuated the increased risk of coronary heart disease[394]. This data suggests that increases in HDL associated ApoE may be a compensatory mechanism of dysregulated lipid metabolism. While more research is necessary, it is possible leucine supplementation in our study positively impacted lipid metabolism, and induced a reduction in HDL associated ApoE. Additionally, at baseline in our study, ApoE was negatively correlated with handgrip-strength on L-HDL (r=-0.484), further supporting a potential benefit of ApoE reduction.

HDL carries a plethora of inflammatory-related proteins that were modified by dietary interventions. For example, LC n-3 PUFA and the maltodextrin control intervention reduced accumulation of SAA2-SAA4 on L-HDL particles. SERPINF2, an acute phase protein[395] and the main plasmin inhibitor[396], were reduced on L-HDL in response to Leu and Leu + n-3 PUFA. High levels of SERPINF2 have been associated with COVID-19[397, 398] and age associated cognitive decline[399] while SERPINF2 deficiency improved cutaneous wound healing[400]. The acute phase protein, SERPINA1[401] was also downregulated by Leu and Leu + LC n-3 PUFA intervention. While this protein is typically upregulated during inflammation[401], it is also recognized as an anti-inflammatory protein[402-406]. However, research shows that SERPINA1 is downregulated on the HDL proteome in response to n-3 PUFA supplementation[379, 407] which was consistent with our findings with Leu ± LC n-3 PUFA. In contrast, Serum amyloid P component (SAP) was upregulated on L-HDL after Leu ± LC n-3 PUFA. SAP is a liver derived pentraxin protein with various roles in the innate immune system[408]. It is a pattern recognition protein that binds to cell debris and apoptotic cells for phagocytosis[409], promotes complement activation[410], and inhibits fibrosis by stimulating IL-10 production from macrophages[411]. Additionally, HDL bound SAP was found to promote SR-B1 mediated cholesterol efflux[412]. A previous study by de Roos *et al.,* showed that SAP decreased in response to n-3 PUFA[407] suggesting it is the leucine component of our intervention that is the main driver of this increase. SAP has previously been shown to positively correlate with BMI, insulin, glucose, triglycerides, CRP, and IL-6 in a large cohort of older participants (*n*=1,599)[413]. Notably, in our study, BMI significantly increased in the Leu and Leu + LC n-3 PUFA groups post-supplementation, which may have contributed to the increase in SAP.

We finally sought to explore how changes in HDL associated proteins over the course of the dietary interventions (across all groups combined) may relate to changes within measures of muscle strength

over the course of the intervention to determine whether the HDL proteome may provide mechanistic insights into disease pathophysiology. We again demonstrated that changes in different proteins tended to correlate with the different measures of strength. Changes in complement C3 (r=-0.472), Coagulation factor II (F2) (r=0.579) and XII (F12) (r=0.506), and ApoD (r=0.427) on S-HDL positively correlated with changes in handgrip strength over the intervention, but not with other anthropometric measures. Changes in levels of IGs on s-HDL (IGLV3-21 (r=-0.589), IGHV3-7 (r=-0.529), IGKV1-17 (r=-0.594) on S-HDL negatively correlated with handgrip strength with no significant correlation to other anthropometric measures. Changes in SERPIND1 (r=0.437), IGLV3-25 (r=-0.578), IGLV1-47 (r=-0.542), IGLC7 (r=-0.541) and QSOX1 (r=-0.517) on S-HDL particles significantly correlated with changes in leg-strength but not the other metrics of strength. Notably SERPIND1 is a liver derived, serine protease inhibitor[414] with anti-thrombin[415] and pro-angiogenic activity[416]. Levels of the protein reportedly decrease with age[417, 418] and negatively correlate with carotid artery plaque thickness[418] and vulnerability to rupture[419]. There were less correlations evident between changes in the protein composition of L-HDL particles and changes in strength. The main correlations evident were changes in A2M (r=-0.572), ALB (0.498), PROS1 (r=-0.427), IGLC3 (r=-0.421) and C3/C5 convertase (r=0.486) on L-HDL which significantly correlated with measures of leg-strength only. A2M is an acute phase protein[420] and broad spectrum protease inhibitor[421]. It acts as an extracellular chaperone, increasing the clearance of misfolded proteins[422] and some cytokines[423, 424] while protecting others from degradation[425]. Increased A2M has been associated with age in rats[426] and humans[427, 428] and is also a biomarker of cardiovascular risk[427, 429]. Interestingly, A2M mRNA was also upregulated in malnourished rats but this decreased after refeeding[430]. PROS1 is an anti-coagulant protein that binds to and inhibits factor IXa[431] and acts as a cofactor for protein C[432]. PROS1 can also inhibit TLR mediated interferon alpha (IFNα) production in dendritic cells[433] and TNF-α, IL-6 and IL-1β in macrophages[434]. Conversely, its production is increased in response to IL-6[435]. Therefore, the increase in HDL associated PROS1 during the intervention, in participants who lost leg-strength, could be a protective mechanism in a bid to dampen an inflammatory response.

These findings demonstrate that the HDL proteome is tracking distinct changes in biology that underpin handgrip strength versus leg-strength and that there are potentially different metabolic phenotypes underpinning different measures of strength. Indeed, changes in handgrip strength over the study did not correlate with changes in leg-strength indicative that these are separate biological entities (Supplement Table 4). Indeed, baseline HS-CRP negatively correlated with changes in leg-strength (r=-0.491) but not handgrip-strength or muscle mass (Supplementary table 3). Shokri-mashhadi et al., similarly demonstrated that CRP negatively correlated with muscle strength but not muscle mass in a systematic review and meta-analysis of *n*=19 cross-sectional studies (*n*=14,650)[436].

This indicates that inflammation is a major driver of leg-strength loss. Indeed, the significant negative correlation between HDL-associated SAA2-4 and leg-strength, but not handgrip-strength, would further confirm this strong link between inflammation and leg-strength.

This was a secondary analysis of a previously completed nutrition intervention study in elderly patients at high-risk of sarcopenia where there was no significant difference in strength after the intervention which limits our capacity to identify key features of responders/non-responders to the interventions. Additionally, this study lacks a group of participants with fully developed sarcopenia. This would allow us to further explore the full spectrum of sarcopenia and provide key insights into the disease trajectory and potential biomarkers of each stage. This was an exploratory study by nature and the findings will need to be validated in a larger separate cohort. The findings were predominantly derived from mass spectrometry analysis of HDL-associated proteins. Additional validation of these findings on other platforms would be beneficial to confirm the findings. A strength of this study was the repeated measures which allowed us to look at changes in the HDL proteome over time and how these changes correlated with strength and muscle mass, rather than relying on a static measurement. A second major strength was the separate analysis of L-HDL and S-HDL particles which provided much clearer insights in the association between their individual protein cargos and clinical parameters pre- and post- dietary intervention.

In conclusion, this study demonstrates that the HDL proteome serves as a nutritionally modifiable biomarker for sarcopenia risk in healthy, community-dwelling older individuals. We have shown that specific HDL proteins correlate with different measures of strength (handgrip and leg) and SMI with a particularly strong negative association between accumulation of inflammatory proteins (eg SAA2-SAA4) and leg-strength. These findings demonstrate that there are different metabolic signatures underpinning these particular anthropometric measurements that were captured within HDL proteomic signatures. We have furthermore demonstrated that the HDL proteome is an extremely sensitive biomarker to track changes in both metabolism and inflammation in response to nutritional interventions. Most notably, the anti-inflammatory (reduced AHSG,PROS1,SERPINA1) and metabolic (reduced apolipoproteins) effects of LC n-3 PUFA were captured within the HDL proteome. Measuring the protein cargo on HDL, and not just static measurements of cholesterol, is thus a potentially powerful approach to track the pleiotropic effects of dietary interventions on health and provides a novel approach to guide precision nutrition approaches to improve health.

Supplementary material

Supplementary Table 1: All proteins identified as significant post-intervention on Control, Leu, and Leu + n-3 PUFA S-HDL.

	Control			Leu			Leu + n-3 PUFA		
	Pre	**Post**	**p-value**	**Pre**	**Post**	**p-value**	**pre**	**post**	**p-value**
AHSG	26.47	25.26	0.0977	26.21	25.18	0.0567	25.96	24.72	0.0075
APOA1	30.04	30.01	0.8522	30.07	30.01	0.3515	30.12	29.82	0.0184
APOA2	27.79	27.11	0.1634	28	27.3	0.0178	27.77	27.1	0.0902
APOD	25.45	25.16	0.0127	25.43	25.24	0.188	25.12	25	0.7244
APOM	25.62	24.85	0.0337	25.33	24.54	0.1374	24.78	25.09	0.502
C1RL	21.56	21.01	0.0824	21.71	21.09	0.0104	21.42	20.77	0.0856
C2	22.78	22.39	0.1603	22.6	22.09	0.0074	22.7	21.96	0.0655
C3	29.91	30.11	0.0001	30.02	30.15	0.1302	30	30.06	0.533
C3/C5	27.69	27.84	0.2516	27.33	27.73	0.0087	27.61	27.2	0.4433
C4A	26.08	26.33	0.0357	26.65	26.64	0.9592	26.48	27.06	0.0989
C5	25.39	25.83	0.0057	25.43	25.8	0.0376	25.47	25.98	0.0008
C6	24.82	24.48	0.0324	24.7	24.62	0.6311	24.76	24.97	0.3015
CFHR1	24.72	24.29	0.2216	24.3	23.8	0.0381	24.56	24.39	0.7702
CFHR2	22.9	23.51	0.4148	23.6	23.44	0.2992	23.05	21.16	0.02
F12	23.21	21.39	0.031	22	21.84	0.7265	22.05	21.47	0.116
GPLD1	20.07	21	0.0231	20.16	19.75	0.4179	20.22	20.84	0.1208
GSN	25.38	24.71	0.0372	24.83	24.79	0.9282	25.15	24.46	0.0065
HPX	28.45	28.2	0.0058	28.35	28.28	0.7297	28.31	27.77	0.2144
IGHA2	25.31	19.7	0.0092	NA	NA	NA	NA	NA	NA
IGHG1	29.53	29.77	0.0278	29.69	29.98	0.1504	29.59	29.71	0.464
IGHG2	27.88	28.6	0.0268	28.1	28.23	0.4598	28.09	28.08	0.9287
IGHV1-18	21.76	23.7	0.1447	22.4	23.82	0.0164	21.65	22.84	0.0697
IGHV1-2	26.5	26.92	0.1539	25.7	26.38	0.0263	25.47	26.2	0.0193
IGHV1-69	26.14	26.66	0.2156	25.89	26.74	0.0009	25.61	26.48	0.0026
IGHV2-70D	23.29	24.53	0.1308	23.12	24.32	0.0496	22.47	23.06	0.4185
IGHV3-49	23.9	24.57	0.0947	23.7	24.36	0.0012	23.68	24.47	0.0032
IGHV3-7	27.87	28.53	0.045	27.82	28.31	0.075	27.93	28.01	0.6922
IGHV4-38-2	23.62	25.35	0.1304	24.24	25.65	0.0459	24.27	25.49	0.063
IGHV5-51	25.88	26.34	0.2134	26.34	26.73	0.0157	25.67	26.11	0.2384
IGKC	29.1	29.62	0.0057	29.22	29.58	0.0098	29.14	29.41	0.1805
IGKV1-6	20.5	22.36	0.0021	19.91	21.84	0.0043	20.72	22.02	0.08
IGKV1-8	NA	NA	NA	21.01	22.51	0.0384	21.39	22.73	0.0199
IGKV2-30	23.96	25.65	0.1077	25.21	25.96	0.003	25.05	24.97	0.9033
IGKV3-20	25.76	26.56	0.1488	25.82	26.72	0.0045	26.14	26.38	0.4049
IGKV6D-21	NA	NA	NA	20.74	20.57	0.7041	19.89	20.93	0.0046

IGLC3	28.54	29.1	0.02	28.58	28.99	0.007	28.5	28.98	0.0363
Ig-like	26.48	27.07	0.039	26.61	27.29	0.0307	26.4	26.13	0.7896
IGLL5	24.33	24.97	0.063	24.51	24.92	0.0464	24.36	24.68	0.5089
IGLV1-51	20.18	22.4	0.0122	21.25	22.33	0.0293	NA	NA	NA
IGLV4-69	NA	NA	NA	21.68	20.64	0.0205	21.23	20.19	0.0386
IGLV7-46	NA	NA	NA	23.47	21.51	0.0338	NA	NA	NA
IGLV8-61	NA	NA	NA	NA	NA	NA	20.64	22.27	0.0153
ITIH4	27.26	27.01	0.0474	27.15	26.79	0.0307	27.27	26.8	0.0159
KRT2	20.66	21.62	0.0313	NA	NA	NA	NA	NA	NA
PGLYRP2	24.43	24.08	0.0435	24.22	24.28	0.7952	24.09	23.58	0.2804
PON1	25.98	25.45	0.3083	25.87	25.25	0.054	25.65	25.28	0.04
PROC	20.59	19.79	0.0009	20.59	19.8	0.0043	20.62	19.99	0.046
SERPINA1	25.05	24.93	0.5187	24.94	24.51	0.1457	25.32	24.76	0.0125
SERPINF2	24.94	24.18	0.0653	24.7	24.03	0.0145	24.64	23.75	0.0449
TF	26.99	27.23	0.0401	26.67	27.18	0.1872	26.77	26.83	0.8635
VTN	27.57	27.3	0.033	27.23	27.19	0.8449	27.35	26.94	0.0736

Supplementary Table 2: All proteins identified as significant post-intervention on Control, Leu, and Leu + n-3 PUFA L-HDL.

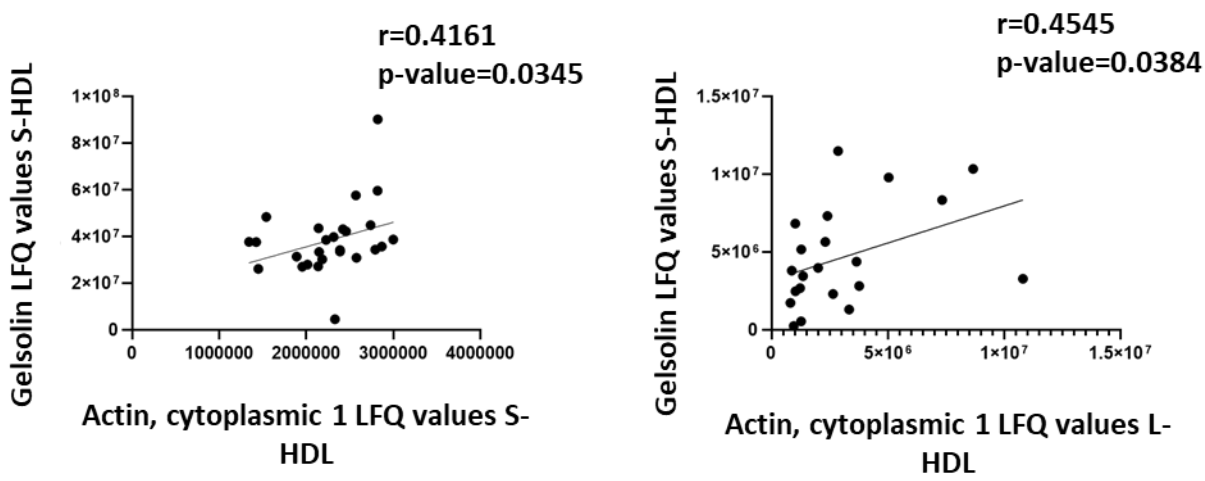
	Control			**Leu**			**Leu + n-3 PUFA**		
	Baseline	**Post**	**p-value**	**Baseline**	**Post**	**p-value**	**Baseline**	**Post**	**p-value**
A1BG	NA	NA	NA	19.18	21.07	0.0008	19.1	20.75	0.0375
A2M	25.15	25.81	0.1281	24.7	25.55	0.0128	25.42	25.25	0.7556
ACTB	20.94	19.48	0.0476	21.32	19.9	0.0076	20.33	19.44	0.0177
APCS	20.68	23.21	0.0931	21.22	24.04	0.0001	21.18	23.79	0.0002
APOA1	29.06	29.02	0.9224	29.1	29.15	0.8772	30.02	29.49	0.0034
APOA2	28.17	27.55	0.2938	26.54	26.83	0.8064	28.23	26.91	0.001
APOA4	26.66	27.29	0.4862	26.74	27	0.6152	27.29	26.43	0.0061
APOB	21.99	22.62	0.339	21.96	21.48	0.4685	22.76	21.9	0.0408
APOC1	25.47	24.72	0.2908	24.83	24.19	0.5115	25.12	23.97	<0.0001
APOD	26.53	26.34	0.6551	26.84	26.25	0.1721	27.25	25.96	0.0003
APOE	26.81	26.6	0.5423	26.51	25.72	0.002	26.81	25.71	0.0161
APOF	20.88	24.13	0.1085	19.9	22.7	0.0045	21.83	23.1	0.1033
APOL1	25.12	25.17	0.6278	25.16	24.85	0.1975	25.04	23.94	0.039
ATRN	20.71	21.6	0.3467	19.76	20.91	0.0081	20.49	20.76	0.5252
C1R	23.66	24.52	0.1055	22.79	24.12	0.0445	23.97	23.42	0.3553
C1S	28.49	28.08	0.2189	28.26	27.86	0.098	28.48	27.02	0.003
C3/C5	22.13	22.62	0.6412	21.95	22.83	0.0408	21.35	22.39	0.0858
C4A	29.03	29.72	0.2916	28.73	29.92	0.009	29.45	29.1	0.3529
C4B	22.7	26.37	0.0257	21.99	26.55	0.002	24.54	26.81	0.0204
C5	26.79	27.51	0.2411	26.36	27.32	0.044	27.14	26.79	0.0963
C7	25.41	25.34	0.9024	24.8	25.05	0.6543	25.61	24.79	0.0005
CDH13	19.98	20.93	0.0623	19.97	20.45	0.2551	19.38	21.01	0.0005
CFH	23.87	24.28	0.0013	24.04	24.35	0.4143	24.64	24.53	0.5608

CFP	23.03	22.91	0.609	21.87	22.86	0.0594	23.11	22.02	0.0019
COMP	22.24	22.5	0.8776	22.63	21.92	0.4925	23.84	22.17	0.0004
CPN1	23.42	23.02	0.5182	22.6	22.05	0.6016	22.85	21.23	0.0016
F13B	24.12	25.08	0.0881	23.25	25.15	0.0338	24.87	24.5	0.1192
F2	24.2	25.56	0.03	23.79	24.94	0.0087	24.19	24.87	0.041
FBLN1	25.36	24.94	0.561	24.6	24.32	0.7344	25.34	23.25	0.0003
FCGR3A	NA	NA	NA	19.85	21.23	0.0004	19.94	20.94	0.015
GPLD1	23.16	25.3	0.1166	22.24	24.64	0.0061	23.73	24.08	0.5933
GSN	22.17	22.09	0.8078	22.17	20.86	0.0128	20.79	21.27	0.4832
HBB	25.31	24.08	0.1028	25.79	24.98	0.0382	25.11	24.91	0.6913
HGFAC	24.77	25.7	0.1548	24.29	25.47	0.1325	25.46	24.4	0.0457
HPX	23.7	21.31	0.1145	23.9	21.64	0.0034	21.63	22.15	0.6471
IGHG1	28.53	27.8	0.2269	29.28	28.3	0.0367	27.63	28.74	0.0223
IGHG2	26.78	24.15	0.0032	26.55	24.65	0.0133	24.89	26.19	0.1225
IGHM	22.98	24.42	0.028	22.65	24.43	0.035	23.77	24.03	0.5888
IGHV1-18	20.13	23.26	0.0834	NA	NA	NA	20.96	23.05	0.0257
IGHV1-69	21.29	25.55	0.0963	22.82	24.98	0.0216	22.51	25.23	0.0396
IGHV3-15	23.41	23.87	0.3573	22.13	23.63	0.0216	22.81	22.97	0.6347
IGHV3-38	19.61	23.51	0.0035	NA	NA	NA	NA	NA	NA
IGHV3-49	22.74	24.81	0.0354	22.13	23.76	0.1239	22.8	23.84	0.1378
IGHV3-7	25	28.33	0.1769	26.75	28.41	0.0001	27.53	28.14	0.1744
IGHV3-72	25.05	26.87	0.126	25.74	27	0.0018	25.8	27.44	<0.0001
IGHV3-74	24.89	27.19	0.0036	23.57	26.05	0.086	25.04	26.26	0.1888
IGHV3-9	NA	NA	NA	23.75	20.2	0.0009	NA	NA	NA
IGHV5-51	23.41	23.8	0.468	24.05	25.08	0.0523	22.87	25.69	0.0171
IGKC	28.1	27.09	0.1088	28.46	27.6	0.0973	27.55	28.62	0.003
IGKV1-8	21.61	20.22	0.0592	21.68	19.84	0.0169	NA	NA	NA
IGKV1D-33	NA	NA	NA	22.98	22.66	0.7633	21.69	25.8	0.0011
IGKV2-30	NA	NA	NA	NA	NA	NA	21.68	23.43	0.0364
IGKV2-40	22.32	26.05	0.3356	NA	NA	NA	23.97	27.02	0.0177
IGKV3-20	NA	NA	NA	23.64	20.93	0.0088	NA	NA	NA
IGKV4-1	22.24	25.05	0.1256	21.17	24.23	0.0536	21.51	24.06	0.0062
IGLC3	27.12	28.28	0.0423	27.8	28.63	0.0029	28.79	28.96	0.4089
Ig-like	NA	NA	NA	20.28	25.86	0.0012	20.85	26.17	0.0002
IGLL5	22.31	24.6	0.2741	21.65	25.25	0.0019	24.39	25.25	0.1855
IGLV3-21	23.01	24.49	0.5762	22.93	25.8	0.0269	24.31	24.72	0.6425
ITIH4	25.7	25.2	0.1139	25.44	24.7	0.001	25.25	25.05	0.5342
KLKB1	20.86	20.6	0.7383	19.78	20.35	0.2586	19.59	20.82	0.0021
LUM	22.83	24.48	0.1072	22.47	24.01	0.012	23.13	23.96	0.0167
PON1	25.73	26.14	0.6369	22.33	26.08	0.0065	26.35	26.26	0.7597
PROS1	23.11	22.82	0.6055	22.94	22.34	0.1026	23.27	21.96	0.0001
QSOX1	21.59	20.52	0.0161	NA	NA	NA	20.54	20.95	0.2823
SAA2-SAA4	28.38	27.16	0.0479	26.95	26.9	0.9504	28.08	26.49	0.0001

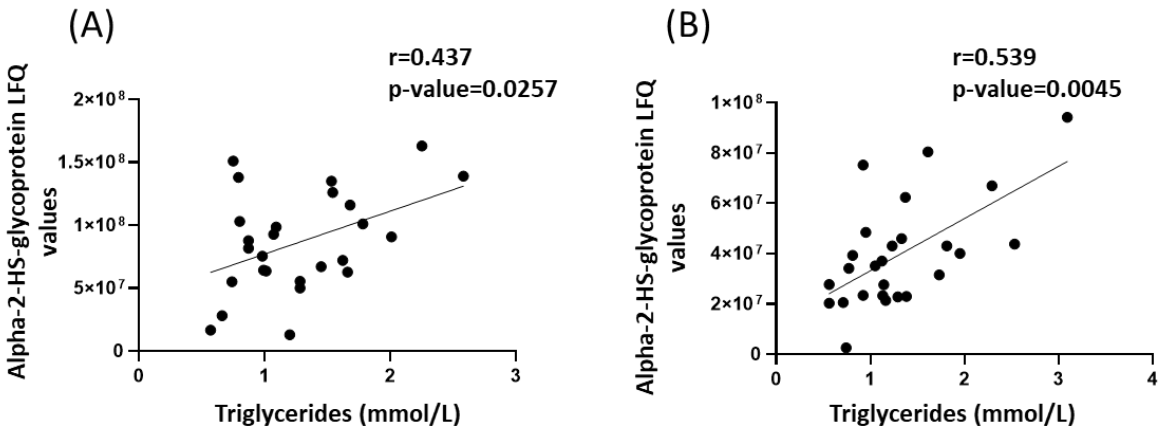
SELENOP	24.1	24.84	0.2059	23.2	24.5	0.064	24.2	23.12	0.0252
SERPINA1	24.13	23.94	0.7564	24.31	23.55	0.018	23.87	23.79	0.8307
SERPINA3	21.83	20.64	0.2582	22.63	20.54	0.0015	21.2	20.9	0.7063
SERPING1	28.29	29.1	0.6119	25.59	29.21	0.0204	29.26	29.02	0.7822
TGFBI	NA	NA	NA	NA	NA	NA	19.75	20.41	0.0137
VASN	19.09	21.47	0.0414	NA	NA	NA	NA	NA	NA

Supplementary Table 3: Correlation of HS-CRP with changes in Handgrip-strength, leg-strength and SMI.

	Δ handgrip-strength (Kg)	**Δ leg-strength (MVC nM)**	**Δ Skeletal muscle index (kg/m^2)**
HS-CRP (mg/L)	0.075	-0.491*	-0.043



Supplementary Figure 1: Correlation of baseline Gelsolin and Actin, cytoplasmic 1 on (A) S- and (B) L-HDL. Spearman correlation analysis was caried out in GraphPad prism on LFQ values.



Supplementary Figure 2 : Correlation analysis of AHSG LFQ values and triglycerides (mmol/L) on S-HDL at (A) baseline and (B) wk24. Correlation analysis carried out in GraphPad prism 10.

Chapter 3:

High Density Lipoprotein proteomic signatures as potential biomarkers of cachexia and sarcopenia in a cohort with gastrointestinal cancer

High Density Lipoprotein proteomic signatures as potential biomarkers of cachexia and sarcopenia in a cohort with gastrointestinal cancer

Rianna McElroy[1], Erin S. Sullivan[2,3,4], Rachel Byrne[5], Aoife Ryan[2,3], Fiona C. McGillicuddy[5], Helen M. Roche[1,6]

1. School of Public Health, Physiotherapy and Sport Science, Conway Institute, Institute of Food and Health, University College Dublin , Dublin, Ireland,

2. School of Food & Nutritional Sciences, College of Science, Engineering & Food Science, University College Cork, Cork, Republic of Ireland,

3. Cork Cancer Research Centre, University College Cork, Cork, Republic of Ireland,

4. Writing Group, The European Union Thematic Network on Integrated Nutrition in Cancer Care (INC2), Croydon, UK

5. Diabetes Complications Research Centre, Conway Institute and School of Medicine, University College Dublin, Dublin 4, Republic of Ireland,

5. Institute for Global Food Security, Queen's University Belfast, Belfast, UK.

***Corresponding Author 1:** Professor Helen M. Roche

Address: UCD Conway Institute, University College Dublin, Belfield, Dublin D04 H1W8,

Ireland

Email: helen.roche@ucd.ie

Telephone: +353 1 716 6845

#Corresponding Author 2: Assistant Professor Fiona C. McGillicuddy

Address: UCD Conway Institute, University College Dublin, Belfield, Dublin D04 H1W8,

Ireland

Email: fiona.mcgillicuddy@ucd.ie

Telephone: +353 1 716 6769

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Abstract

Introduction: Cancer cachexia, with and without features of muscle wasting (sarcopenia), is extremely prevalent in patients with cancer, and significantly increases mortality risk, but is difficult to detect in a timely manner due to a lack of non-invasive biomarkers. Inflammation and impaired metabolism are thought to underpin cachexia and sarcopenia. We therefore hypothesized that the proteins attached to high-density lipoprotein (HDL) particles, which carry both inflammatory and metabolism-related proteins, may serve as novel biomarkers of cachexia and sarcopenia.

Methods: Patients with gastrointestinal (GI) cancer (*n*=84) were classified as having Cancer cachexia (CC) (*n*=47) or cancer without cachexia (C-C) (*n*=26). HDL was isolated from serum using size exclusion chromatography and purified using lipid removal agent prior to trypsin digestion. Peptides were analysed on a timsTOF mass-spectrometry and searched in MaxQuant against the human database. T-tests were carried out in Perseus to determine significant proteins. This process was repeated to determine differences between patients with sarcopenia (C+S) and patients without (C-C). A scoring algorithm based on collation of Z-scores of significantly different proteins was generated to identify panel/s of proteins that could sensitively serve as a biomarker of cachexia and/or sarcopenia. Performance was calculated using receiver operating characteristic (ROC) Area Under the Curve (AUC)

Results: A total of *n*=8 HDL proteins decreased in CC patients, compared to C-C, including Vitronectin (VTN), Beta-Ala-His dipeptidase (CNDP1), Phosphatidylinositol-glycan-specific phospholipase D (GPLD1), Apolipoprotein A-II (ApoA2), Hepatocyte growth factor activator (HGFAC), Apolipoprotein B-100 (ApoB), Apolipoprotein L1 (ApoL1) and SUN domain-containing protein 3 (SUN3) and *n*=5 were increased including Ig Kappa chain C region (IGKC), Thyroxine binding globulin (SERPINA7), Alpha-1-antichymotrypsin (SERPINA3), Ceruloplasmin (CP) and Vitamin K-dependent protein C (PROC). Collation of proteins into a score demonstrated a ROC area under the curve (AUC) of 0.810 (sensitivity=0.702, specificity=0.115) in the ability to differentiate between CC patients vs C-C. A second score was generated based on HDL-proteins that were significantly different in C+S and C-C patients including *n*=9 increased proteins, 78 kDa glucose-regulated protein (HSPA5), Prothrombin (F2), Alpha-2-macroglobulin (A2M), Sex hormone-binding globulin (SHBG), CP, SERPINA3 and SERPINA7 while *n*=7 protein was decreased, CNDP1, GPLD1, ApoA2, SUN3 ApoL1, insulin growth factor-binding protein 3 (IGFBP3) and Selenoprotein P (SELENOP). Collation of proteins into a score demonstrated an ROC AUC of 0.861 (sensitivity=0.813, specificity=0.154) in the ability to differentiate between C+S and C-C.

Conclusion: The HDL proteome is enriched with acute phase proteins , in both cachexia and sarcopenia including SERPINA3, CP and A2M and depleted of Apolipoproteins (ApoL1, ApoA2, ApoB) indicative

that the HDL particle can sense metabolic derangements contributing to significant weight and muscle wasting. The HDL proteome is a potentially novel biomarker of wasting phenotypes in patients with GI cancer.

Introduction

Cancer cachexia is a multifactorial wasting disease characterised by weight loss, muscle atrophy, anorexia, reduced treatment tolerance and shorter survival[2]. Cachexia affects up to 80% of cancer patients with lung, pancreatic and gastrointestinal (GI) cancers being the most affected[1]. While the aetiology of cachexia is not fully understood, lack of appetite, inflammation, lipolysis and increased muscle catabolism with reduced muscle protein synthesis are key contributing factors[437]. Additionally, sarcopenia, or low muscle mass, can exist in conjunction with cachexia or on its own[161]. Patients can lose large amounts of muscle mass but remain weight stable or even gain weight[13, 438]. The majority of patients with cancer are older when diagnosed and may already suffer from age-related sarcopenia that can be further exasperated by tumour-related factors[438]. However, as the muscle status of patients is unknown before diagnosis, it is difficult to decipher whether sarcopenia is caused by the tumour or age-related factors.

Both cachexia and sarcopenia have been associated with poor survival[439], increased treatment-related toxicities[25, 440] and worse surgical outcomes[29, 441]. Differentiating between cachexia and sarcopenia is difficult, and indeed only a few studies have directly compared the effects of cachexia and sarcopenia in a patient cohort[442161, 443]. In patients with gastric cancer (*n*=1215), cachexia was a predictor of reduced survival in later stages of disease, while sarcopenia was a predictor of reduced survival in the early stages of disease. The authors theorised that surgery to remove the tumour can resolve cachexia in the early stages of disease while surgeries tend to be less radical in the more advanced stages[442]. Additionally, patients with cachexia had lower visceral fat when compared to patients with sarcopenia which may leave them with less of an energy reserve under stressful conditions[442]. In patients with pancreatic cancer, cachexia was associated with worse overall survival, while sarcopenia was only associated with poor survival in patients receiving chemotherapy or those who had a high BMI[161]. While there are no effective treatment available for either disorder, it is proposed that a personalised treatment approach indicated based upon the different sub-types of cachexia presentation (with/without muscle loss) may be more effective.

The diagnosis of cachexia remains a challenge. Insufficient awareness, understaffing and a lack of standardised screening tools and definition all contribute to underdiagnosis[33]. The most widely used definition, determined by international consensus in 2011 by Fearon et al., proposes “weight loss >5% over past 6 months (in absence of simple starvation); or BMI <20 and any degree of weight loss >2%; or Appendicular skeletal muscle index consistent with sarcopenia (males <7·26 kg/m²; females <5·45 kg/m²) and any degree of weight loss >2%”[2]. While this definition is comprehensive, clinically more accurate tools that can identify cachexia, and in particular loss of skeletal muscle mass, could improve

diagnosis, to prioritise nutritional support to positively impact quality of life. Accurate and repeated weight monitoring is a challenge; also accurate skeletal muscle measurement requires costly and time-consuming methods, impeding thorough clinical assessment. Ideally, a non-invasive clinical biomarker is needed for the routine screening of patients with cancer that can enable early identification and prioritisation of those most in need of dietician referral[444].

While several biomarkers of cachexia exist in the literature, none have been translated to the clinic due in part to issues around reproducibility. For example, interleukin-6 (IL-6), a pro-inflammatory cytokine, has been identified as a potential biomarker for weight loss and muscle mass in cancer in some studies[96, 175, 445, 446] but not in others[68, 183, 447]. Transforming growth factor- beta (TGF-β) family members such as activin-A, myostatin[448, 449] and growth/differentiation factor 15 (GDF-15)[448] have also been identified as potential biomarkers. GDF-15 acts on the glial-cell-line-derived neurotrophic factor family receptor α–like (GFRAL) receptor in the hindbrain inducing anorexia[450] but has also been shown to act on skeletal muscle in mice by maintaining energy expenditure during calorie restriction[448]. It is a stress-related molecule[450] and while it has been associated with cachexia[451], it has also been identified as a biomarker for cancer detection[452], diabetes[453] and cardiovascular disease[454], and thus lacks specificity. Specificity is a challenge when it comes to biomarker discovery. To overcome this, our approach combines the expression of multiple proteins into a single score rather than relying on the measurement of one single protein. Cachexia biomarker discovery to date has predominantly focused on analysis of invasive (but specific) host and tumour tissue biopsies or less invasive (but non-specific) serum/plasma matrices[61]. Enriching serum for 'cancer biomarker-containing' fractions such as exosome isolation has received increasing attention in the biomarker field in recent years to overcome specificity issues with serum/plasma matrices[455, 456], however large starting materials are generally required for exosome isolation[457] and the exosomes are highly likely to be cancer rather than cachexia specific[455]. High-density lipoprotein (HDL) particles are complex emulsions of lipids, proteins and metabolites that are predominantly derived from metabolic tissues including the liver (70%) and small intestine (30%)[296] and are intricately involved in modulating cardiometabolic health[298]. HDL carries a suite of metabolic (apolipoproteins AI, AII, AIV, CI-CIII, E, M) and pro- and anti-inflammatory proteins including clusterin, haptoglobin, serum amyloid A (SAA), and complement factors as well as anti-oxidant proteins including paraoxonase-1 (PON-1)[311] illustrating that HDL quality may be an important sensor for a wide set of metabolic and inflammatory disease states, including cachexia[458, 459]. HDL particles are enriched with pro-inflammatory proteins and depleted of antioxidant and anti-inflammatory proteins in the setting of obesity, independent of changes in HDL-cholesterol (HDL-C) levels[297]. In addition, the HDL proteome could specifically track changes in expression of metabolic and inflammatory proteins in the liver in response to an anti-inflammatory pharmacotherapy intervention

in preclinical obesity studies[296], demonstrating successful enrichment of liver-derived proteins by analysing the HDL proteome[296]. Alteration in liver metabolism and inflammation is increasingly recognised as a potential contributor to cachexia[460-462]. Activation of the acute phase response (APR), futile cycling of glucose and lactate[461,463], alteration of liver metabolome[101], mitochondrial alterations[460], and inflammation-induced cholestasis[100] have all been identified in preclinical models of cancer cachexia. Within the current study, we hypothesized that the HDL particle represents an important, readily accessible circulating molecule, whose cargo of metabolic, inflammatory and oxidative proteins may serve as a proxy for GI-related cancer cachexia. Importantly for biomarker discovery, HDL particles can be isolated from small volumes of serum, derived from a minimally invasive blood-test, resulting in enrichment for multiple immune-metabolic proteins.

This study determined if the HDL proteome may act as a biomarker of cachexia and sarcopenia, in a GI cancer cohort. The primary aim was to determine whether general cachexia (with or without sarcopenia) resulted in a unique HDL proteomic signature that could be exploited for diagnostic purposes. The second aim was to determine whether different HDL proteomic signatures may be evident in patients with sarcopenia relative to those without. Our findings indicate profound changes in the HDL proteome in the setting of cachexia and sarcopenia, with enrichment of proinflammatory acute phase proteins (APP) such as ceruloplasmin, and Alpha-1-antichymotrypsin and depletion of apolipoproteins such as Apolipoprotein B-100 and Apolipoprotein L1. Thus the HDL proteome may serve as a novel and innovative biomarker of high-risk patients with cancer cachexia.

Methods

Study population:

This study was approved by the Clinical Research Ethics Committee of the Cork Teaching Hospitals. Patients in this study are a subgroup of a larger prospective observational study in a cohort of ambulatory oncology patients receiving Systemic Anti-Cancer Therapy, designed to determine the impact of abnormal body composition phenotypes on survival, conducted by the University College of Cork (UCC), as previously described[464]. Data was collected from adults >18 years of age with a solid malignancy of the foregut who were well enough to receive chemotherapy and gave written consent. Data collection took place between June 2012 and September 2016 in Cork University Hospital or the Mercy University Hospital. All patients were receiving anti-cancer treatment at the time of recruitment and were not asked to fast prior to blood sample being taken; treatment cycle and types are outlined in supplementary table 3 & 4. For the current study, *n*=84 patients with gastro-oesophageal (*n*=44) or colorectal cancer (*n*=40) were selected for HDL proteomics analysis. Cachexia was determined using the Fearon[2] definition of cachexia "weight loss >5% over past 6 months (in absence of simple starvation); or BMI <20 and any degree of weight loss >2%; or Appendicular skeletal muscle index consistent with sarcopenia (males <7·26 kg/m²; females <5·45 kg/m²) and any degree of weight loss >2%"[2]. Sarcopenia was determined using the Martin[465] definition of sarcopenia; men: if BMI <24.99 than a SMI of 43 cm^2/m^2 is used, if BMI >25 than a SMI of <53 cm^2/m^2 is used, women: <41 cm^2/m^2 for all BMI. Skeletal muscle area (cm^2) was assessed via CT scan at the 3rd lumbar vertebrae which was then normalised by height to determine Skeletal muscle index (SMI)[464].

HDL isolation by Fast Protein Liquid Chromatography (FPLC):

Serum (100µl) was separated using x2 sequential superose 6 Increase 10/300 GL columns (Cytiva) linked to a GE ÄktaFPLC system (GE healthcare). Resulting HDL fractions were pooled and enriched for lipid-containing particles by incubation with lipid removal agent (100mg/ml). The resulting pellet was washed twice in 50mM Ammonium bicarbonate (ABC) and then resuspended in 2M urea made up in 50mM ABC.

Trypsin digestion:

5mM of Dithiothreitol (DTT) was added to samples to reduce the disulphide bonds in proteins and incubated for 30 minutes at 60°C. 10mM Iodoacetamide (IAA) was added to alkylate the cysteine residues and incubated in the dark at room temperature for 30 minutes. Samples were diluted with 50µl of ABC to reduce urea to a working range for the trypsin. 0.5µg of trypsin, using Trypsin singles (Sigma-Aldrich), was added to each sample and incubated overnight at 37°C with shaking. Formic acid (1% final concentration) was added to inhibit the trypsin and centrifuged for 5min at 10,000 RPM.

Supernatant containing peptides was transferred to a fresh tube and peptides were loaded onto C18 Ziptips and eluted in buffer containing 70% acetonitrile and 0.1% formic acid. Samples were dried down in a Concentrator 5301 (Eppendorf) and resuspended in 3% Acetonitrile with 0.1% formic acid. Peptides were quantified by Denovix DSC spectrometer (Denovix) at 205/215nm.

Mass spectrometry on a timsTOF:

Evotips (EVOSEP) were loaded as per manufacturer's instructions with 700ng of peptides from each sample. Samples were run on a timsTOF Pro mass spectrometer (Bruker Daltonics, Bremen, Germany) coupled to the EvoSep One system (EvoSep BioSystems, Odense, Denmark). Peptides were separated on a reversed-phase C18 Endurance column (15cm x 150µm ID) over 44 minutes using a flow rate of 0.5ul/min and an increasing concentration of formic acid in acetonitrile. TIMS (Trapped Ion Mobility Spectrometry) and PASEF (Parallel Accumulation Serial) modes were enabled on the timsTOF pro and operated in a positive ion polarity. Spectra was captured in the mass range from 100 to 1,700 m/z. Each PASEF cycle took 1.17 s and consisted of one MS ramp for precursor detection followed by 10 PASEF MS/MS ramps. Peptides were run on the TimsTOF by Dr. Catriona Scaife in the UCD proteomics core.

Proteomic data analysis:

Raw Label Free Quantification (LFQ) values were analysed in Perseus (version 1.6.15.0). Contaminants, proteins only identified by site and reverse database identifications were filtered from the dataset. A valid value filter of 70% in at least one group was applied. Proteins were LOG2 transformed and missing values imputed from the normal distribution. A paired t-test with a p-value of 0.05 was carried out comparing Cachexia vs Control and Sarcopenia vs Control. Results were normalised using z-score and visualised as a heat map. Raw LFQ values were graphed in GraphPad prism (version 10) and significance determined using an independent t-test on normally distributed data or a Mann-Whitney U test on data that was not normally distributed. Correlation analysis was carried out on raw LFQ values in GraphPad prism using Pearson correlation for normally distributed data and Spearman correlation for data that was not normally distributed. Normality was determined using the Shapiro-Wilk test of normality.

Statistical analysis:

Data analysis of patient characteristics was carried out in SPSS (version 27). Continuous variables were analysed using an independent samples t-test if they had a normal distribution or a Mann-Whitney U if not normally distributed. A chi-square test was used to determine significance of categorical data.

Score creation:

A z-score was created for each significant protein per subject. The z-scores for the proteins that decreased were combined for each patient and the composite score was subtracted from the collation of the z-scores from the proteins that increased generating a cachexia score. This was repeated for the sarcopenia score. Score performance was evaluated in SPSS (version 27) using a Receiver Operating Curve (ROC). Performance was assessed by measuring Area Under the Curve (AUC) with a ROC AUC of >0.8 considered good and >0.9 considered excellent for a diagnostic performance[466]. A score of 0.5 indicates no discrimination while a score >0.9 indicates outstanding performance.

Generation of modified Glasgow prognostic score (mGPS) and Global Leadership Initiative on Malnutrition (GLIM) criteria :

Two scores have been proposed for routine cachexia screening, the inflammation based mGPS and body composition based GLIM criteria. We therefore applied these to our patient cohort to compare against our novel proteomics-based risk scores. The mGPS score is based on measures of albumin and CRP with a score of 0 given to patients with CRP $\leqslant$ 10 mg/l and albumin $\geqslant$35 g/l, a score of 1 to patients with CRP > 10 mg/l and a score of 2 given to patients with CRP >10 mg/l and albumin <35 g/l[467]. The GLIM criteria is a 2 step process in which patients must have 1 phenotypic feature (>5% weight loss in last 6 months, BMI <20 < 70 years, or <22 if > 70 years, or reduced muscle mass) and 1 etiological criteria (inflammation, reduced food intake or disorder affecting absorption) to be diagnosed as malnourished – notably this is a binary score. To evaluate the GLIM criteria we chose the Martin[465] definition of sarcopenia to define low muscle mass in our cohort while we used the BMI cut-offs suggested by the GLIM criteria to define a low BMI. Patients with a CRP >10 mg/l were considered inflamed. For the etiological criteria, reduced food intake or a condition causing malabsorption was determined if the patients reported reduced food intake or had diarrhoea, constipation, nausea or low appetite.

Results

Phenotypic characteristics of cachexia:

Characteristics of the patients derived from the prospective observational cohort of ambulatory oncology patients receiving Systemic Anti-Cancer Therapy designed to determine the impact of abnormal body composition on survival[464], are summarised in Table 1. The cohort consisted of both gastro-oesophageal (*n*=44) and colorectal cancer (*n*=40) patients, in order to have a near homogenous GI cancer focus for HDL proteomics analysis. In total, *n*=47 patients met the criteria for cancer cachexia (C+C) and *n*=26 did not (cancer without cachexia (C-C)). The groups were largely age- and sex-matched with greater proportion of males within this study. While not significant, there were a greater number of gastroesophageal patients in the CC group (67%) compared to cancer patients without cachexia (C-C) (38.5%) Clinical parameters often employed to identify cancer cachexia, such as C-reactive protein (CRP), albumin and handgrip strength were not significantly different between the patients with/without cachexia. Indeed CRP was elevated in both groups. The CC group lost 9.81 ± 0.8 Kg in the previous 6 months, while cancer patients without cachexia were weight stable. At the time of survey, 14.9% of patients with cachexia were classified as underweight, according to BMI, while none of the control group were underweight. The majority of subjects with cachexia had a normal BMI (46.8%), while the majority of the cancer patients without cachexia were overweight (46.2%). 26.9% of the control group were identified as having obesity versus 6.4% of the cachexia group. SMI was also significantly different between the 2 groups ($p<0.01$).

Table 1: Patient characteristics of subjects with Cancer without Cachexia versus Cancer with Cachexia

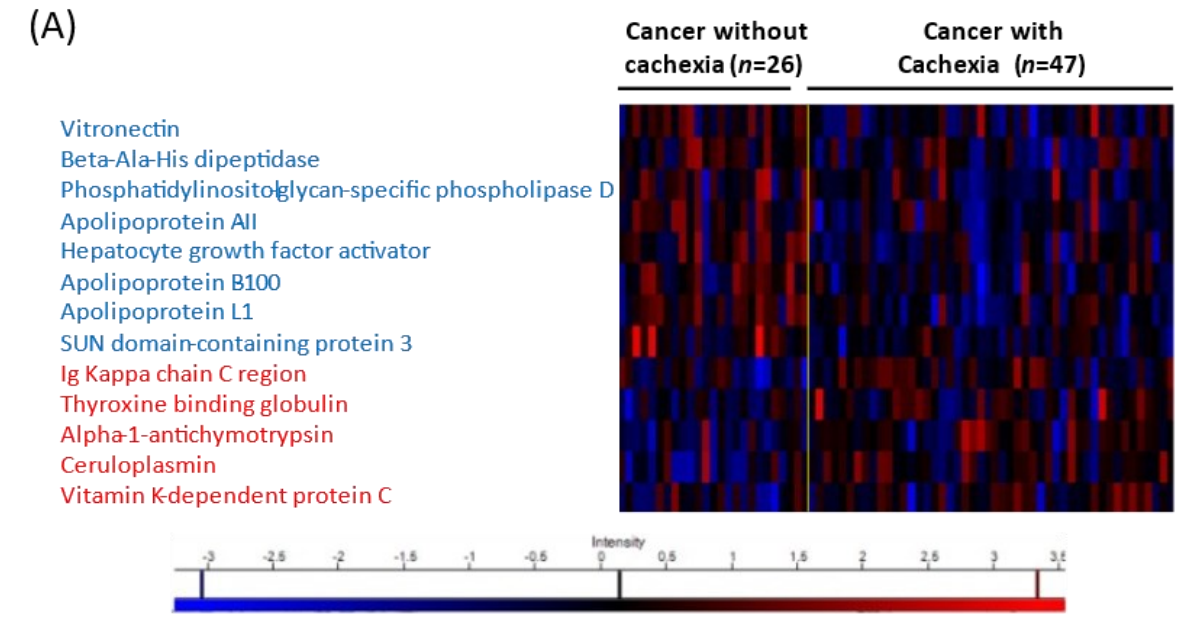
		Cancer without cachexia (n=26)	Cancer Cachexia (n=47)	p-value
Sex (female %)		34.60%	25.50%	0.431
Age (years)		63.18 ± 1.8	63.31 ± 1.4	0.955
Height (meters)		1.68 ± 0.02	1.71 ± 0.01	0.179
Cancer group	Gastro-oesophageal	38.50%	61.70%	0.086
	Colorectal	61.50%	38.30%	
Weight at survey (kg)		78.87 ± 2.86	70.97 ± 2.07	0.027
Pre diagnosis weight (kg)		81.39 ± 3.16	79.24 ± 2.36	0.588
6 months weight change (%)		0.69 ± 0.74	-9.81 ± 0.8	<0.0001
Body Mass Index (BMI) at survey		27.87 ± 0.99	24.04 ± 0.56	0.001
BMI Category	Underweight BMI	0.00%	14.90%	0.046
	Normal BMI	26.90%	46.80%	0.135
	Overweight BMI	46.20%	31.90%	0.312
	Obese BMI	26.90%	6.40%	0.028
Skeletal Muscle Area (cm^2)		155.64 ± 6.02	140.78 ± 4.84	0.054
Skeletal Muscle Index (cm^2)/(m^2)		54.58 ± 1.52	47.6 ± 1.42	0.002
Muscle attenuation (HU)		36.63 ± 1.64	38.93 ± 0.93	0.192
Total adipose tissue area (cm^2)		367.87 ± 41.02	312.7 ± 22.93	0.207
Metastasis		38.50%	42.60%	0.807
Stage 4		46.20%	42.60%	0.809
ECOG score	0	34.60%	42.60%	
	1	42.30%	42.60%	
	2	23.10%	14.90%	0.639
Hand grip (kg)		26.39 ± 1.86	28.72 ± 1.5	0.342
C-reactive protein (mg/L)		13.5 ± 5.48	11.72 ± 2.44	0.676
Albumin (g/L)		34.46 ± 0.71	33.81 ± 0.5	0.543
Lymphocytes (cell count x10^9/L)		1.36 ± 0.16	1.12 ± 0.09	0.143
Neutrophils (cell count x10^9/L)		3.83 ± 0.35	3.41 ± 0.23	0.293
Neutrophil to lymphocyte ratio		3.74 ± 0.54	4.03 ± 0.47	0.814
White cell count (cell count x10^9/L)		5.95 ± 0.52	6.61 ± 1.33	0.587
Hemoglobin (g/dl)		11.91 ± 0.28	11.74 ± 0.27	0.675
Currently smoking		11.50%	4.30%	0.34
Currently drinking		34.60%	44.70%	0.463

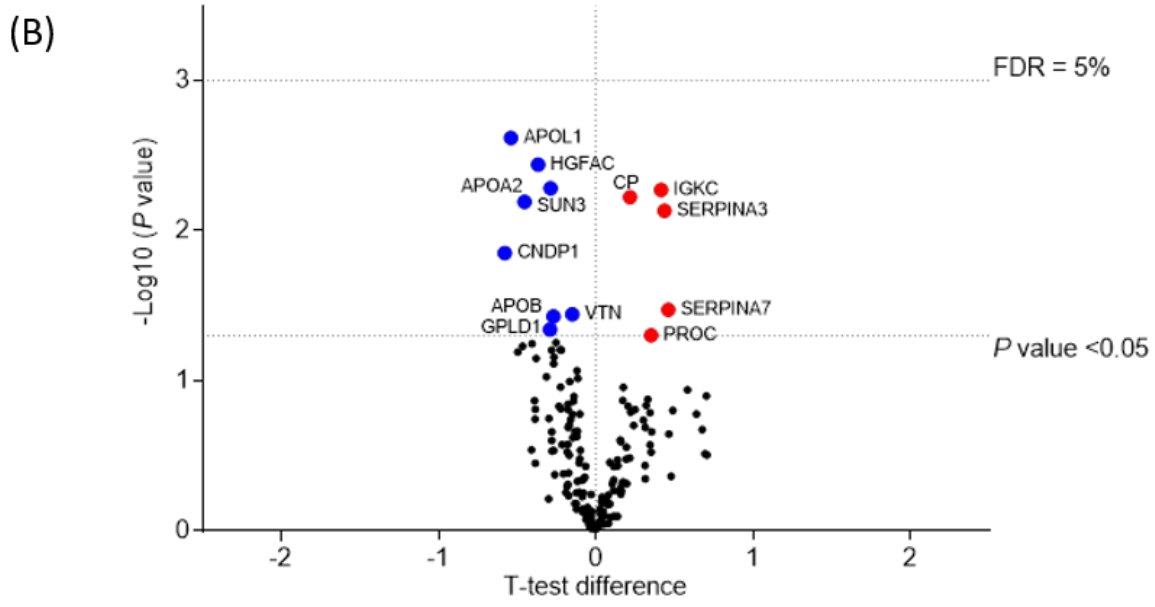
Continuous variables are presented as mean ± standard error while categorical values are presented as percentages. Continuous variables were analysed by one-way ANOVA with Bonferroni post-hoc analysis or Kruskal Wallis test. Repeated Mann Whitney-U tests were performed in a post-hoc manner

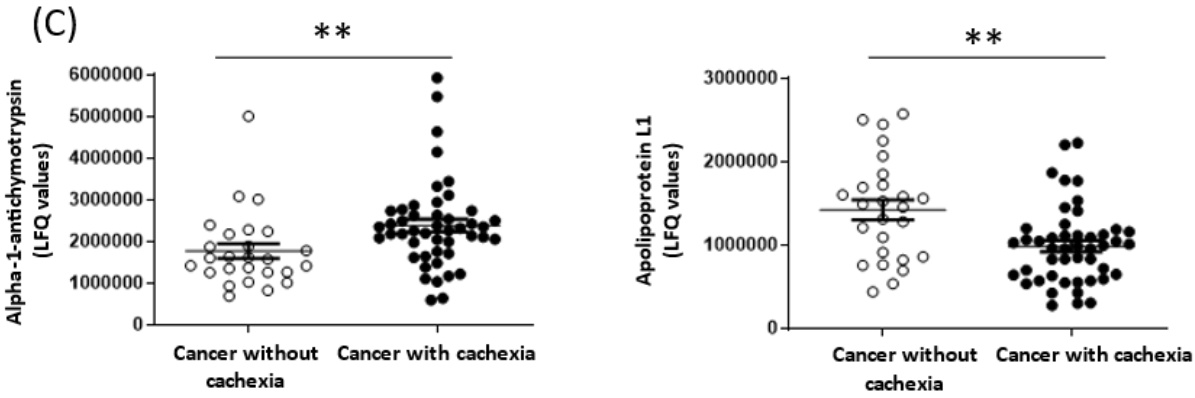
if a significant Kruskal Wallis test was found. Categorical variables were analysed using a chi-square t-test. P-value is noted as not significant (ns) unless a significant result was found.

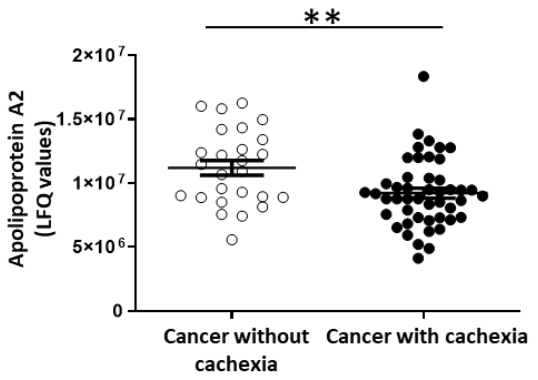
The HDL proteome is affected by the presence of cancer cachexia:

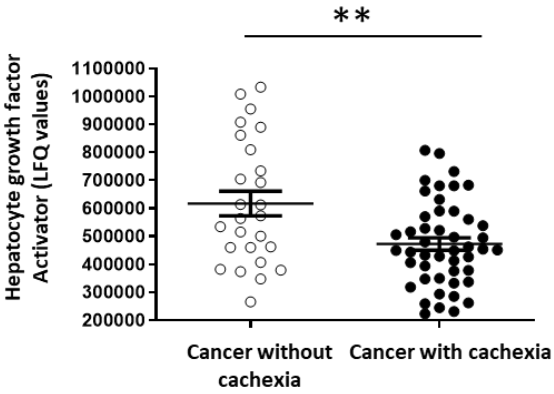
Mass spectrometry analysis identified *n*=172 proteins within HDL-containing FPLC fractions (Supplementary table 5). In total, *n*=13 proteins were significantly different between the CC and C-C (Figure 1). Of these, *n*=8 proteins were significantly decreased in the CC versus C-C group including Vitronectin (VTN), Beta-Ala-His dipeptidase (CNDP1), Phosphatidylinositol-glycan-specific phospholipase D (GPLD1), Apolipoprotein A-II (ApoA2), Hepatocyte growth factor activator (HGFAC), Apolipoprotein B-100 (ApoB), Apolipoprotein L1 (ApoL1) and SUN domain-containing protein 3 (SUN3) and *n*=5 proteins were significantly increased including Ig Kappa chain C region (IGKC), Thyroxine binding globulin (SERPINA7), Alpha-1-antichymotrypsin (SERPINA3), Ceruloplasmin (CP) and Vitamin K-dependent protein C (PROC).

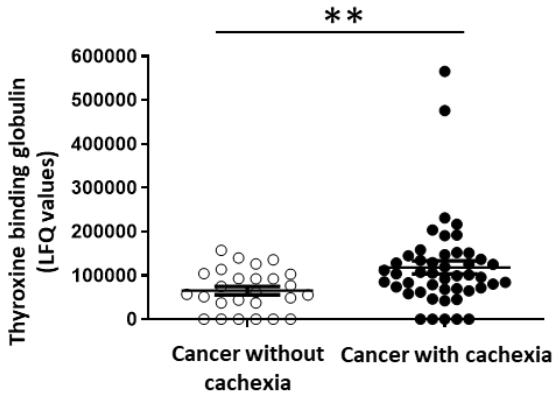

(A)
Cancer without cachexia (n=26)
Cancer with Cachexia (n=47)
Vitronectin
Beta-Ala-His dipeptidase
Phosphatidylinositol-glycan-specific phospholipase D
Apolipoprotein AII
Hepatocyte growth factor activator
Apolipoprotein B100
Apolipoprotein L1
SUN domain-containing protein 3
Ig Kappa chain C region
Thyroxine binding globulin
Alpha-1-antichymotrypsin
Ceruloplasmin
Vitamin K-dependent protein C
Intensity

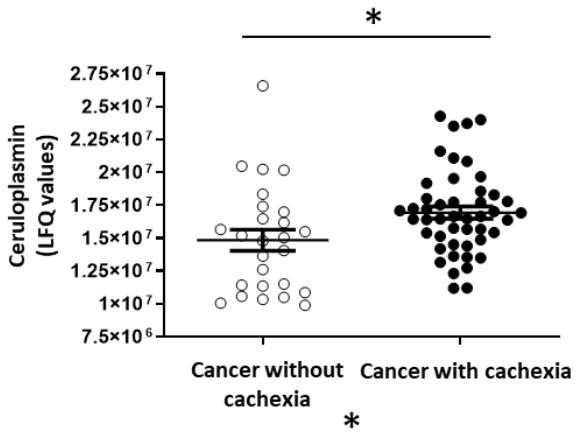

(B)
FDR = 5%
P value <0.05
-Log10 (P value)
T-test difference
APOL1
HGFAC
APOA2
SUN3
CP
IGKC
SERPINA3
CNDP1
APOB
GPLD1
VTN
SERPINA7
PROC

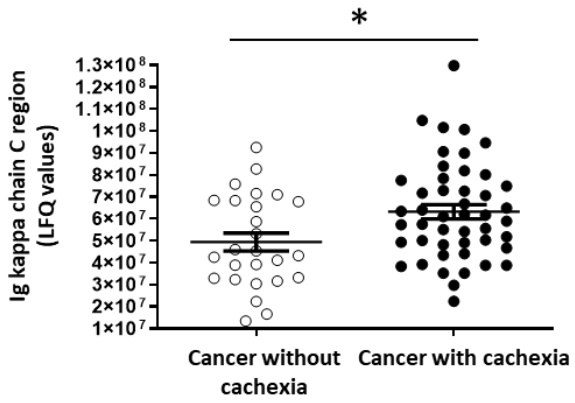

(C)
**
Alpha-1-antichymotrypsin (LFQ values)
Cancer without cachexia
Cancer with cachexia
**
Apolipoprotein L1 (LFQ values)
Cancer without cachexia
Cancer with cachexia

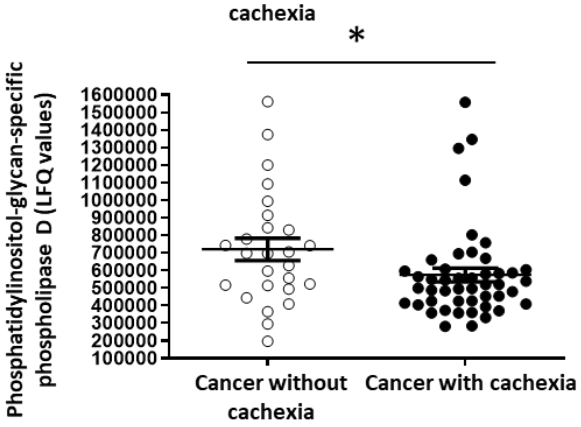

**
Apolipoprotein A2 (LFQ values)
2×10^7
1.5×10^7
1×10^7
5×10^6
0
Cancer without cachexia
Cancer with cachexia

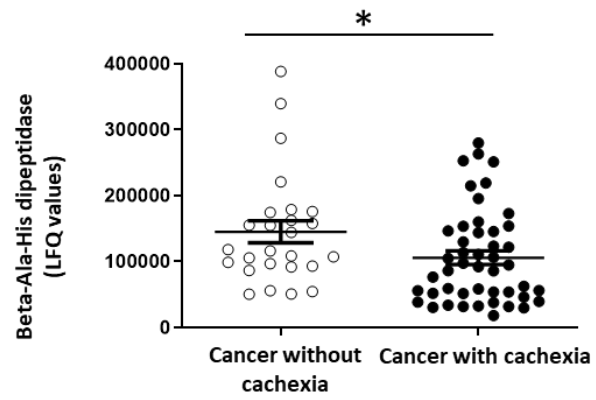

**
Hepatocyte growth factor Activator (LFQ values)
1100000
1000000
900000
800000
700000
600000
500000
400000
300000
200000
Cancer without cachexia
Cancer with cachexia

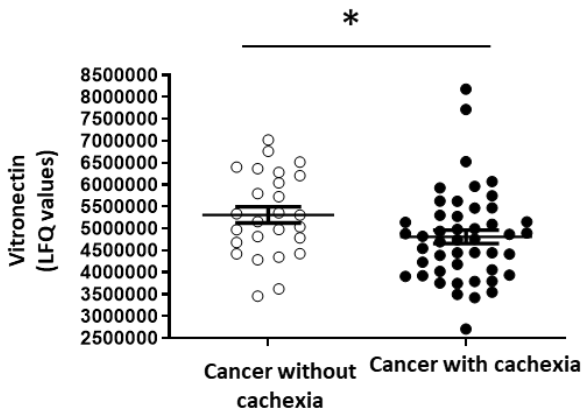

**
Thyroxine binding globulin (LFQ values)
600000
500000
400000
300000
200000
100000
0
Cancer without cachexia
Cancer with cachexia


*
Ceruloplasmin (LFQ values)
2.75×10^7
2.5×10^7
2.25×10^7
2×10^7
1.75×10^7
1.5×10^7
1.25×10^7
1×10^7
7.5×10^6
Cancer without cachexia
Cancer with cachexia


*
Ig kappa chain C region (LFQ values)
1.3×10^8
1.2×10^8
1.1×10^8
1×10^8
9×10^7
8×10^7
7×10^7
6×10^7
5×10^7
4×10^7
3×10^7
2×10^7
1×10^7
Cancer without cachexia
Cancer with cachexia


*
Phosphatidylinositol-glycan-specific phospholipase D (LFQ values)
1600000
1500000
1400000
1300000
1200000
1100000
1000000
900000
800000
700000
600000
500000
400000
300000
200000
100000
Cancer without cachexia
Cancer with cachexia


*
Beta-Ala-His dipeptidase (LFQ values)
400000
300000
200000
100000
0
Cancer without cachexia
Cancer with cachexia


*
Vitronectin (LFQ values)
8500000
8000000
7500000
7000000
6500000
6000000
5500000
5000000
4500000
4000000
3500000
3000000
2500000
Cancer without cachexia
Cancer with cachexia

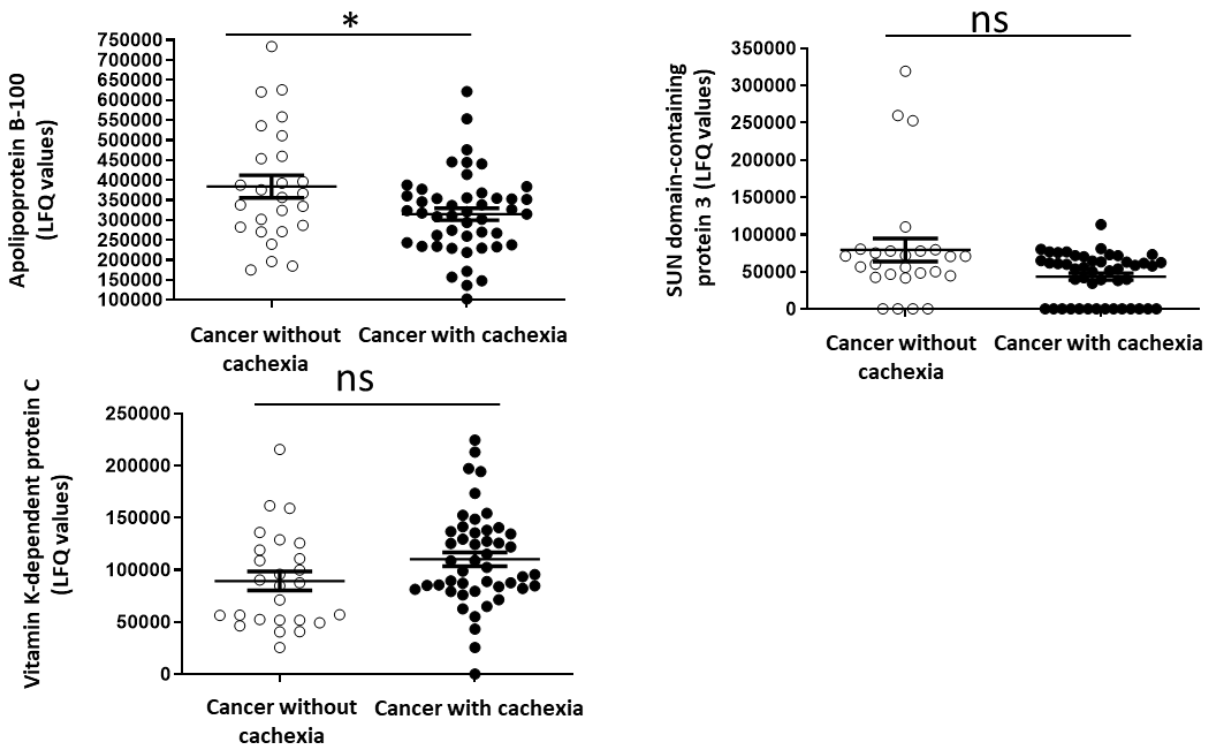
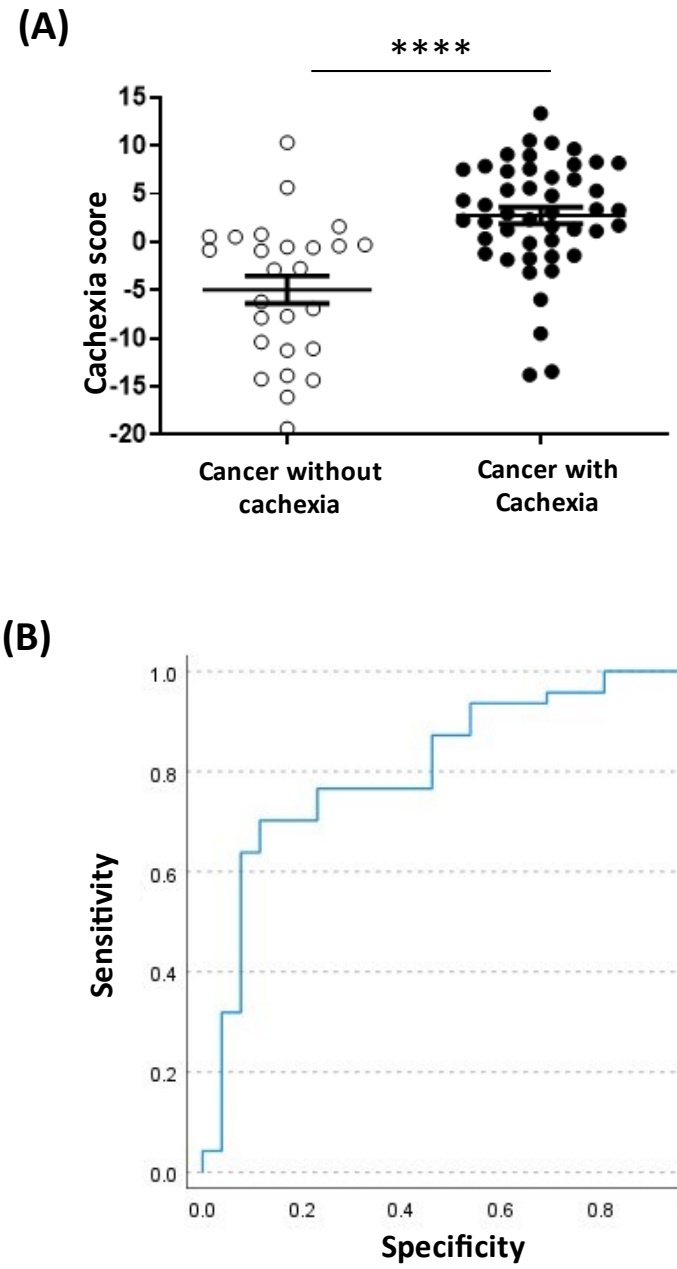


Figure 1: (A) Heatmap, (B) Volcano plot and (C) Dot plots of proteins significantly affected by CC in a gastrointestinal cohort. Heatmap was generated using the significant proteins as identified by t-test in Perseus. The –Log(p-value) and t-test difference determined in Perseus were used to plot the volcano plot for visualisation. Red data points indicate proteins upregulated in CC and blue indicate proteins downregulated. Horizontal dashed lines on the heatmap indicate significance (<0.05) and false discovery rate (5%). Dot plots show raw LFQ values without normalisation and imputation. Significance of LFQ values was determined in GraphPad prism for dot plots.p**<0.01,p*<0.05, ns = not significant. Vitamin K-dependent protein C (PROC), SUN domain-containing protein 3 (SUN3), Apolipoprotein L1 (APOL1), Ceruloplasmin (CP), Alpha-1-antichymotrypsin (SERPINA3), Ig kappa chain C region (IGKC), Apolipoprotein A-II (APOA2), Vitronectin (VTN), Apolipoprotein B-100 (APOB), Thyroxine-binding globulin (SERPINA7), Phosphatidylinositol-glycan-specific phospholipase D (GPLD1), Hepatocyte growth factor activator (HGFAC), Beta-Ala-His dipeptidase (CNDP1).

A score created from HDL-associated proteins has a higher performance than individual proteins for the identification of cachexia:

Given the complexity of the development of CC, we hypothesised that collation of key differentiated proteins into a single risk score would outperform any individual protein's ability to identify cachexia. The *n*=13 proteins that were significantly different between the cachexia and control groups (Figure *1*) were collated into a cachexia score (Figure *2*A). Dot plots of LFQ values are shown (Figure *1*C) as the score is made using a z-score summation of proteins. This score had an ROC AUC of 0.810, indicating an excellent score (Figure *2*B) ($p<0.0001$). The AUC of each individual protein was also evaluated (Table *2*) SERPINA3 had an AUC of 0.720 followed by ApoL1 with an AUC of 0.712. All other proteins had an AUC between 0.637 and 0.692.

A cut-off value for the cachexia score was determined using coordinates of the ROC curve. A cut-off value of 0.96 was suggested by the Kolmogorov-Smirnov (K-S) metric. Patients with a score above this value were identified as having cachexia. This value has a sensitivity of 0.702 and a specificity of 0.115. Sensitivity represents the likelihood of a true positive test result and specificity represents the probability of a false positive[468]. A sensitivity closer to 1 and a specificity closer to 0 is ideal[468]. Using this cut-off value, 33 out of 47 (70.2%) subjects within the CC group were correctly identified as having cachexia. In the C-C, 3 out of 26 (11.5%) were false positives and therefore misclassified as having cachexia. Interestingly, *n*=1 patient who was misclassified by our score had a CRP of 125mg/L indicating they were highly inflamed. Another patient who was misclassified as having cachexia just missed the cut-off point for being diagnosed with sarcopenia.



AUC	Cut-off point	Sensitivity	Specificity
0.810	0.96	0.702	0.115

Figure 2: (A) Dot plot of the cachexia score (B) ROC curve of cachexia score generated in SPSS. Cachexia score was generated as a z-score summation of significant proteins as identified by t-test. The scores performance was determined by ROC curve in SPSS. The AUC was 0.810 and a cut-off point of 0.96 was selected. This cut-off point has a sensitivity of 0.702 and a specificity of 0.115. p****<0.0001

Table 2: AUC of proteins significantly affected by CC compared to C-C.

Protein	AUC
Apolipoprotein L1	0.712
Apolipoprotein A2	0.692
Hepatocyte growth factor activator	0.692
Sun-domain containing 3	0.637
Vitronectin	0.658
Apolipoprotein B-100	0.646
Phosphatidylinositol-glycan-specific phospholipase D	0.673
Beta-Ala-His dipeptidase	0.660
Ceruloplasmin	0.681
Alpha-1-antichymotrypsin	0.720
Thyroxine-binding globulin	0.690
Vitamin-K dependent protein C	0.641
Ig-Kappa C region	0.669

AUC of proteins significantly affected by cachexia. AUC was determined by ROC analysis in SPSS.

Performance of the novel proteomics cachexia score compared to current state-of-the-art screening tools: mGPS and GLIM, in our cohort:

To further test the performance of our proteomics-based cachexia score, we evaluated two proposed cachexia screening tools, mGPS and GLIM in these patients. While not currently used clinically for the detection of cachexia, both have been evaluated for their potential to identify patients with cancer cachexia[35, 159]. The GLIM criteria has the benefit of being very comprehensive with a 2-step scoring system while the mGPS has the benefit of being weight-independent and based on circulating biomarkers. The GLIM criteria correctly identified 80.4% of the CC group as 'malnourished'; while identifying 100% of those in the C-C group as 'not malnourished' (Table 5). The mGPS exhibited the poorest performance across the 3 scores. 27.7% of the CC group had a mGPS score >1, indicative of cachexia, while 19.2% of the C-C group had a score >1 (Table 3). The AUC of the GLIM criteria was 0.904 which is considered excellent and the AUC of the mGPS was 0.536, which is considered to be of no diagnostic value. It is notable that weight-loss was one of the key criteria for the classification of cachexia within our study, and is also a key factor within the GLIM score and thus there is a significant positive confounder favouring this particular score.

Table 3: Comparison of the cachexia score to 2 proposed screening tools for patients with cancer, mGPS and GLIM.

		Cancer without cachexia	**Cancer with cachexia**	**AUC**
Cachexia score	*Cancer without cachexia*	*88.5%*	*29.8%*	*0.810*
	Cancer with cachexia	*11.5%*	*70.2%*	
mGPS	*Score 0*	*80.8%*	*72.3%*	*0.536*
	Score >1	*19.2%*	*27.7%*	
GLIM	*No Malnutrition*	*100%*	*19.1%*	*0.904*
	Malnutrition	*0%*	*80.4%*	

Percentage of patients diagnosed as at risk according to each score. Modified Glasgow prognostic score (mGPS) relies on measurements of CRP and albumin. Global Leadership Initiative on Malnutrition

(GLIM) is a 2-Step score relying on the presence of one phenotypic feature (weight loss, low BMI etc.,) and one etiological criteria (anorexia, inflammation etc.,)

Phenotypic characteristics of patients with cachexia and sarcopenia:

Among the patients with cancer cachexia, *n*=21 also met the Martin[465] definition of sarcopenia (Cachexia with sarcopenia (C+S)) while *n*=26 did not (cachexia without sarcopenia (C-S))(Table *4*). There were significantly fewer females in the C-S group (7.7%) than in the C+S group (47.6%) ($p<0.01$). Groups were well-matched in all other parameters. SMI was significantly different between the C+S group (40.41 ± 1.42 cm^2/m^2 versus C-S group (53.42 ± 1.54 cm^2/m^2) as well as versus the C-C (54.58 ± 1.52 cm^2/m^2) ($p<0.001$) . There were significantly more patients classified as underweight by BMI in the C+S group (28.6%) than in the C-Sarcopenia (3.8%) and C-C group (0%) ($p<0.01$).

Table 4: Patient characteristics-Exploring sarcopenia within the cachexia definition

		Cancer without cachexia (*n*=26)*a	Cachexia without sarcopenia (*n*=26)*b	Cachexia with sarcopenia (*n*=21)*c	p-value
Sex (female %)		34.60%	7.70%	47.60%	*b,c=0.008
Age (years)		63.18 ± 1.8	62.36 ± 2	64.49 ± 1.94	0.744
Height (meters)		1.68 ± 0.02	1.71 ± 0.02	1.71 ± 0.02	0.432
Cancer group	Gastro-oesophageal	38.50%	57.70%	66.70%	
	Colorectal	61.50%	42.30%	33.30%	0.132
Weight at survey (kg)		78.87 ± 2.86	74.29 ± 2.41	66.86 ± 3.4	*a,b=0.015
Pre diagnosis weight (kg)		81.39 ± 3.16	82.33 ± 2.73	75.42 ± 3.99	0.299
6 months weight change (%)		0.69 ± 0.74	-9.36 ± 0.82	-10.37 ± 1.49	*a,b=0.001, *a,c=0.001
Body Mass Index at survey		27.87 ± 0.99	25.21 ± 0.68	22.58 ± 0.86	*a,c<0.001
BMI Category	Underweight BMI	0.00%	3.80%	28.60%	*a,c=0.002
	Normal BMI	26.90%	53.80%	38.10%	0.138
	Overweight BMI	46.20%	34.60%	28.60%	0.441
	Obese BMI	26.90%	7.70%	4.80%	*a,c=0.048
Skeletal Muscle Area (cm^2)		155.64 ± 6.02	157.42 ± 5.26	120.18 ± 6.28	*a,c<0.001, *b,c<0.001
Skeletal Muscle Index (cm^2)/(m^2)		54.58 ± 1.52	53.42 ± 1.54	40.41 ± 1.42	*a,c<0.001, *b,c<0.001
Muscle attenuation (HU)		36.63 ± 1.64	39.78 ± 1.27	38.03 ± 1.36	0.304
Total adipose tissue area (cm^2)		367.87 ± 41.02	355.4 ± 31.55	259.84 ± 30.24	0.079
Metastasis		38.50%	42.30%	42.90%	0.809
Stage 4		46.20%	42.30%	42.90%	0.799
ECOG score	0	34.60%	42.30%	42.90%	
	1	42.30%	42.30%	42.90%	
	2	23.10%	15.40%	14.30%	0.509
Hand grip (kg)		26.39 ± 1.86	30.39 ± 1.92	26.52 ± 2.33	0.271
C-reactive protein (mg/L)		13.5 ± 5.48	14.04 ± 3.63	8.86 ± 3.08	0.387

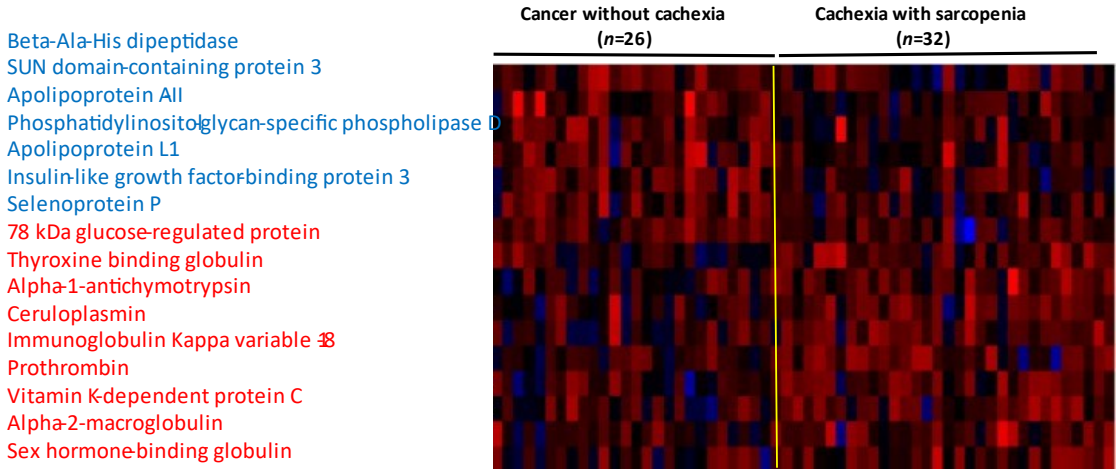
Albumin (g/L)	34.46 ± 0.71	34.08 ± 0.6	33.48 ± 0.85	0.634
Lymphocytes (cell count x10^9/L)	1.36 ± 0.16	1.1 ± 0.13	1.15 ± 0.12	0.332
Neutrophils (cell count x10^9/L)	3.83 ± 0.35	3.4 ± 0.27	3.41 ± 0.39	0.577
Neutrophil to lymphocyte ratio	3.74 ± 0.54	4.15 ± 0.52	3.87 ± 0.88	0.426
White cell count (cell count x10^9/L)	5.95 ± 0.52	5.31 ± 0.36	8.41 ± 3.13	0.806
Hemoglobin (g/dl)	11.91 ± 0.28	11.68 ± 0.36	11.81 ± 0.41	0.888
Currently smoking	11.50%	3.80%	4.80%	0.320
Currently drinking	34.60%	46.20%	42.90%	0.428

Continuous variables are presented as Mean ± standard error while categorical values are presented as percentages. Continuous variables were analysed by one-way ANOVA with Bonferroni post-hoc analysis or Kruskal Wallis test. Repeated Mann Whitney-U tests were performed in a post-hoc manner if a significant Kruskal Wallis test was found. Categorical variables were analysed using a chi-square t-test. p-value is noted as not significant (ns) unless a significant result was found.

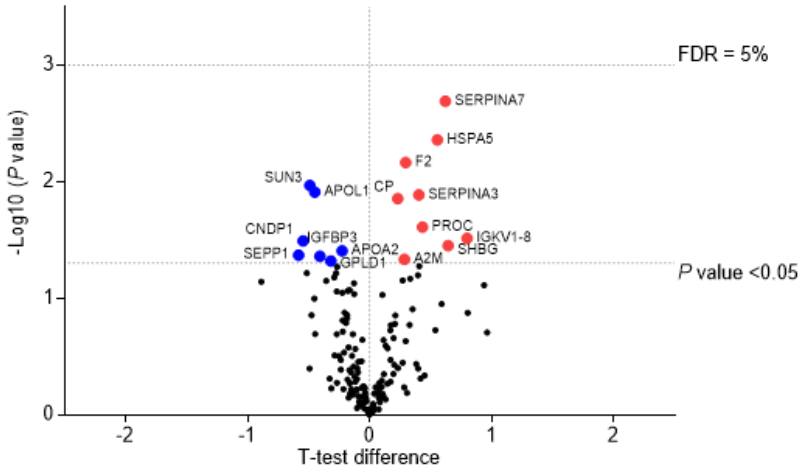
The HDL proteome is modulated in the setting of sarcopenia:

We subsequently investigated whether different HDL-associated proteins could identify patients with sarcopenia versus those without. To this end, all patients who met the criteria for sarcopenia, *n*=32, were compared to the cancer without cachexia (C-C) group (*n*=26) (Figure *3*A). Within these *n*=32 patients were *n*=11 patients who did not meet the definition of cachexia but did have sarcopenia and so are only included in this analysis (For continuity this group will still be referred to as C+S). There were *n*=16 proteins significantly different between the C+S and C-C groups. Of these, *n*=9 proteins were increased in the C+S group including 78 kDa glucose-regulated protein (HSPA5), Prothrombin (F2), Alpha-2-macroglobulin (A2M), Sex hormone-binding globulin (SHBG), Vitamin-K dependent protein C (PROC), Immunoglobulin Kappa Variable 1-8 (IGKV1-8) and SERPINA7 while *n*=7 proteins was decreased, Beta-Ala-His dipeptidase (CNDP1), Apolipoprotein A-II (ApoA2), Phosphatidylinositol-glycan-specific phospholipase D (GPLD1), Apolipoprotein L1 (ApoL1), Insulin-like growth factor-binding protein 3 (IGFBP3), Selenoprotein P (SEPP) and SUN domain-containing protein 3 (SUN3).

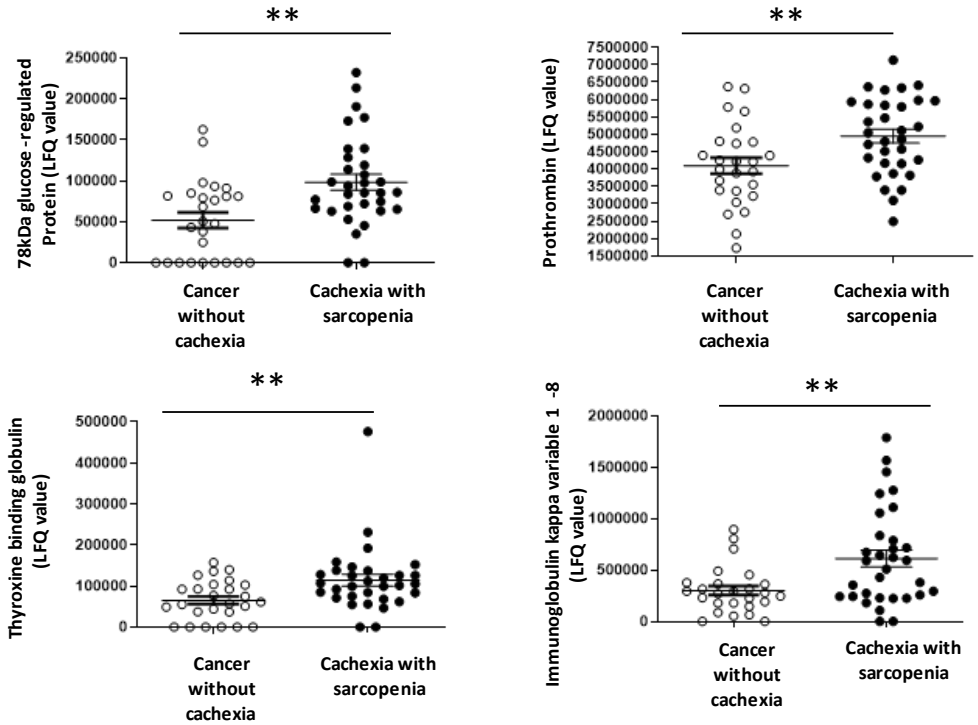
(A)

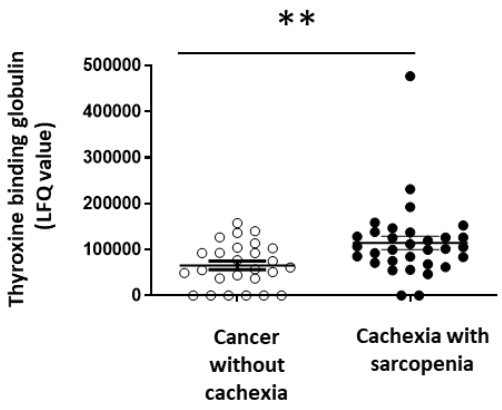

Beta-Ala-His dipeptidase
SUN domain-containing protein 3
Apolipoprotein AII
Phosphatidylinositol-glycan-specific phospholipase D
Apolipoprotein L1
Insulin-like growth factor-binding protein 3
Selenoprotein P
78 kDa glucose-regulated protein
Thyroxine binding globulin
Alpha-1-antichymotrypsin
Ceruloplasmin
Immunoglobulin Kappa variable 1-8
Prothrombin
Vitamin K-dependent protein C
Alpha-2-macroglobulin
Sex hormone-binding globulin
Cancer without cachexia (n=26)
Cachexia with sarcopenia (n=32)
Intensity

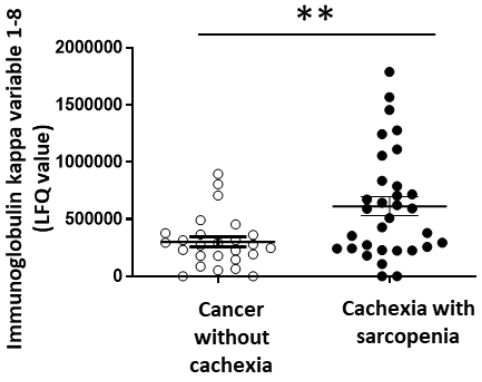
(B)

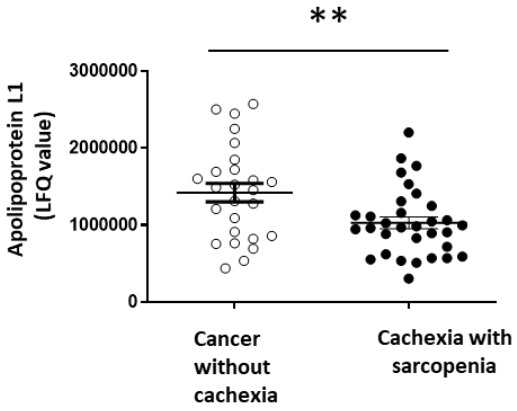

FDR = 5%
P value <0.05
-Log10 (P value)
T-test difference
SERPINA7
HSPA5
F2
SERPINA3
PROC
IGKV1-8
SHBG
A2M
CP
APOL1
SUN3
CNDP1
IGFBP3
SEPP1
APOA2
GPLD1

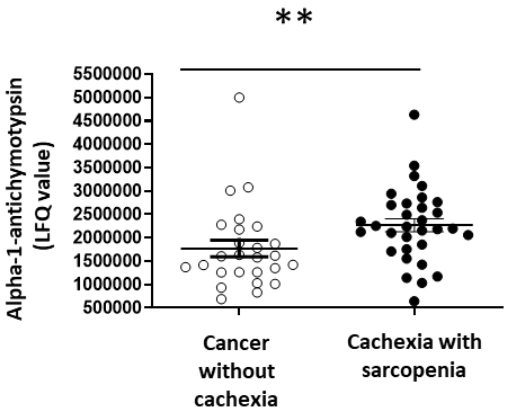
(C)

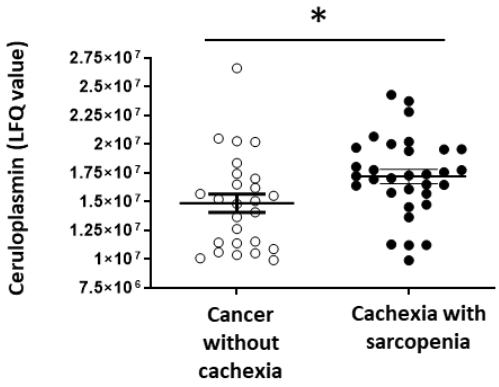

78kDa glucose -regulated Protein (LFQ value)
Prothrombin (LFQ value)
Thyroxine binding globulin (LFQ value)
Immunoglobulin kappa variable 1 -8 (LFQ value)
**
Cancer without cachexia
Cachexia with sarcopenia

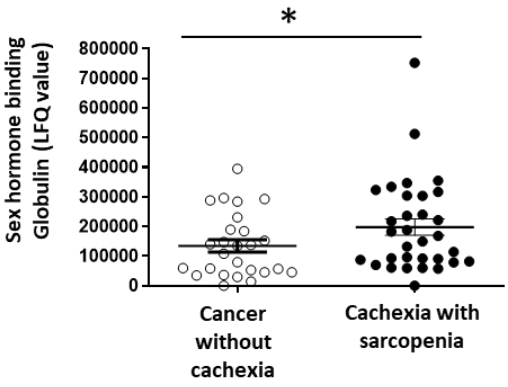

**
Thyroxine binding globulin (LFQ value)
500000
400000
300000
200000
100000
0
Cancer without cachexia
Cachexia with sarcopenia

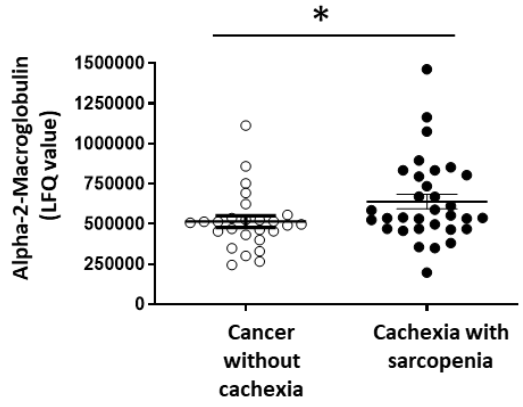

**
Immunoglobulin kappa variable 1-8 (LFQ value)
2000000
1500000
1000000
500000
0
Cancer without cachexia
Cachexia with sarcopenia

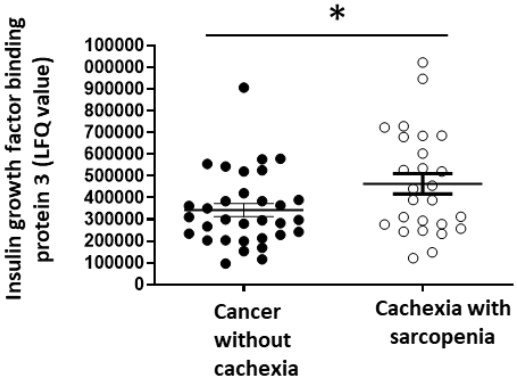

**
Apolipoprotein L1 (LFQ value)
3000000
2000000
1000000
0
Cancer without cachexia
Cachexia with sarcopenia


**
Alpha-1-antichymotypsin (LFQ value)
5500000
5000000
4500000
4000000
3500000
3000000
2500000
2000000
1500000
1000000
500000
Cancer without cachexia
Cachexia with sarcopenia


*
Ceruloplasmin (LFQ value)
2.75×10^7
2.5×10^7
2.25×10^7
2×10^7
1.75×10^7
1.5×10^7
1.25×10^7
1×10^7
7.5×10^6
Cancer without cachexia
Cachexia with sarcopenia


*
Sex hormone binding Globulin (LFQ value)
800000
700000
600000
500000
400000
300000
200000
100000
0
Cancer without cachexia
Cachexia with sarcopenia


*
Alpha-2-Macroglobulin (LFQ value)
1500000
1250000
1000000
750000
500000
250000
0
Cancer without cachexia
Cachexia with sarcopenia


*
Insulin growth factor binding protein 3 (LFQ value)
100000
000000
900000
800000
700000
600000
500000
400000
300000
200000
100000
0
Cancer without cachexia
Cachexia with sarcopenia

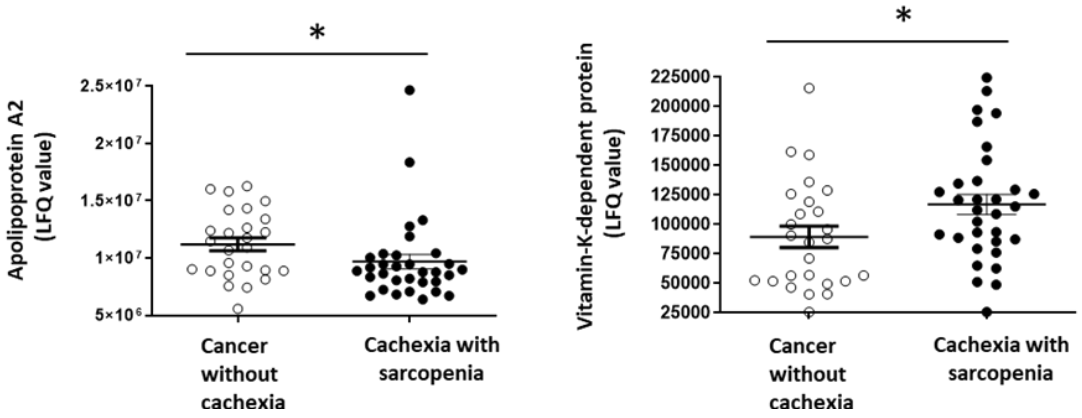
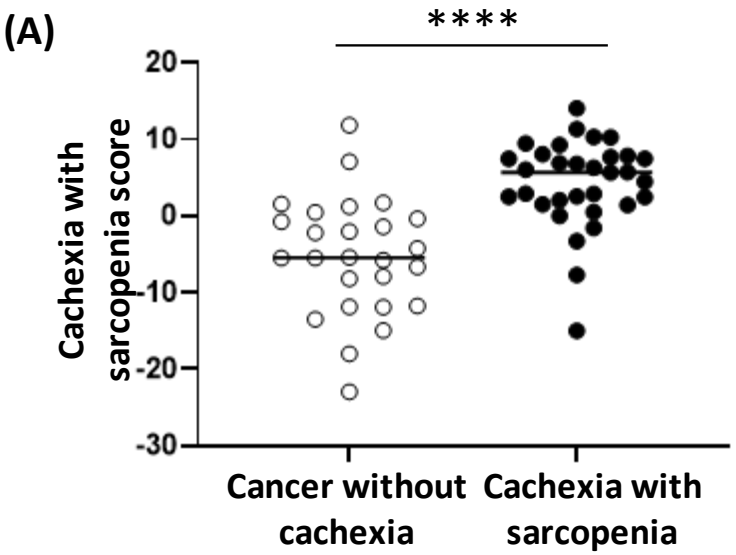
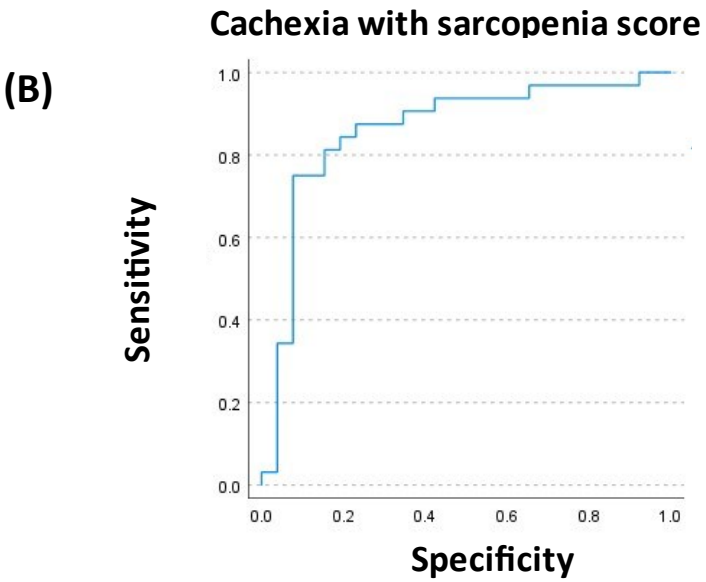


Figure 3: (A) Heatmap, (B) Volcano plot and (C) Dot plots of proteins significantly affected by sarcopenia in a gastrointestinal cohort. Heatmap was generated using the significant proteins as identified by t-test in Perseus. The –Log(p-value) and t-test difference determined in Perseus were used to plot the volcano plot for visualisation. Red data points indicate proteins upregulated in sarcopenia and blue indicate proteins downregulated. Horizontal dashed lines on the heatmap indicate significance (<0.05) and false discovery rate (5%). Dot plots show raw LFQ values without normalisation and imputation. Significance of LFQ values was determined in GraphPad prism for dot plots. ***<0.001, **<0.01,*<0.05, ns = not significant. 78 kDa glucose-regulated protein (HSPA5), Prothrombin (F2), Alpha-2-macroglobulin (A2M), Sex hormone-binding globulin (SHBG), Immunoglobulin kappa variable 1-8 (IGKV1-8), Thyroxine-binding globulin (SERPINA7), Alpha-1-antichymotrypsin (SERPINA3), Beta-Ala-His dipeptidase (CNDP1), Apolipoprotein A-II (ApoA2), Phosphatidylinositol-glycan-specific phospholipase D (GPLD1), Apolipoprotein L1 (ApoL1), Insulin-like growth factor-binding protein 3 (IGFBP3), Selenoprotein P (SEPP) and SUN domain-containing protein 3 (SUN3), Vitamin-K-dependent protein C (PROC) and Ceruloplasmin (CP).

A score created from HDL proteins can identify patients with sarcopenia:

Similar to our cachexia score, we generated a sarcopenia score from all the proteins significantly affected by sarcopenia (Figure 4A). The score had an AUC of 0.861, indicating an excellent performance. A cut-off value for the Sarcopenia score was determined using the coordinates of the ROC curve. A cut-off value of 1.327 was suggested by the Kolmogorov-Smirnov (K-S) metric. Patients with a score above this value were identified as having sarcopenia. This value has a sensitivity of 0.813 and a specificity of 0.154. This score correctly identified *n*=26 of the *n*=32 patients (81.25%) with sarcopenia while *n*=4 out of the *n*=26 patients (15.4%) were false positives. Of these *n*=4 patients, *n*=2 were also misdiagnosed by the cachexia score, *n*=1 of these patients just missed the cut-off point for a sarcopenia diagnosis and *n*=2 had severely elevated CRP. The individual proteins were also evaluated for their ability to predict sarcopenia via ROC AUC (Table 5). HSPA5 had the greatest performance with an AUC of 0.737 followed by SERPINA7 with an AUC of 0.714. CNDP1 performed the worst with an AUC of 0.637 and no protein performed as well as the score. In a subgroup analysis, the sarcopenia score was not significantly different in patients with sarcopenia or cachexia ± sarcopenia, relative to the cachexia alone group. Finally, the novel sarcopenia score negatively correlated with SMI (spearman r= -0.6363, p<0.0001) (Figure 5A) including n=11 of the significant proteins, but to a lesser extent than the combined score. These include, GPLD1 (r=0.4276, p<0.0001), SERPINA3 (r=-0.425, p<0.0001), SEPP1 (r=0.4056, p<0.01), CP (r=-0.4045, p<0.01), IGFBP3 (r=0.392, p<0.01), PROC (r=-0.3739, p<0.01), SHBG (r=-0.3577), p<0.01), A2M (r=-0.3119, p<0.05), F2 (r=-0.302, p<0.50), APOA2 (r=0.2762, p<0.05) and CNDP1 (r=0.2729, p<0.05) (Figure 5B).





AUC	Cut-off point	Sensitivity	Specificity
0.861	1.327	0.813	0.154

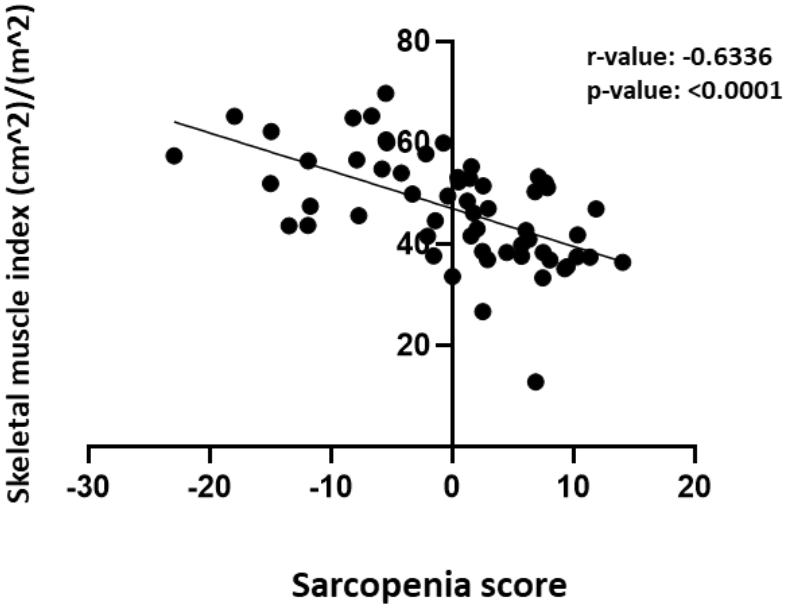
Figure 4: (A) Dot plot of the sarcopenia score composed of the proteins 78 kDa glucose-regulated protein, prothrombin and Alpha-2-macroglobulin. (B) ROC curve of sarcopenia score generated in SPSS. Sarcopenia score was generated as a z-score summation of selected proteins. The scores performance was determined by ROC curve in SPSS. The AUC was 0.796 and a cut-off point of -1.49 was selected. This cut-off point has a sensitivity of 0.813 and a specificity of 0.154. p****<0.0001.

Table 5: AUC of proteins significantly affected by sarcopenia

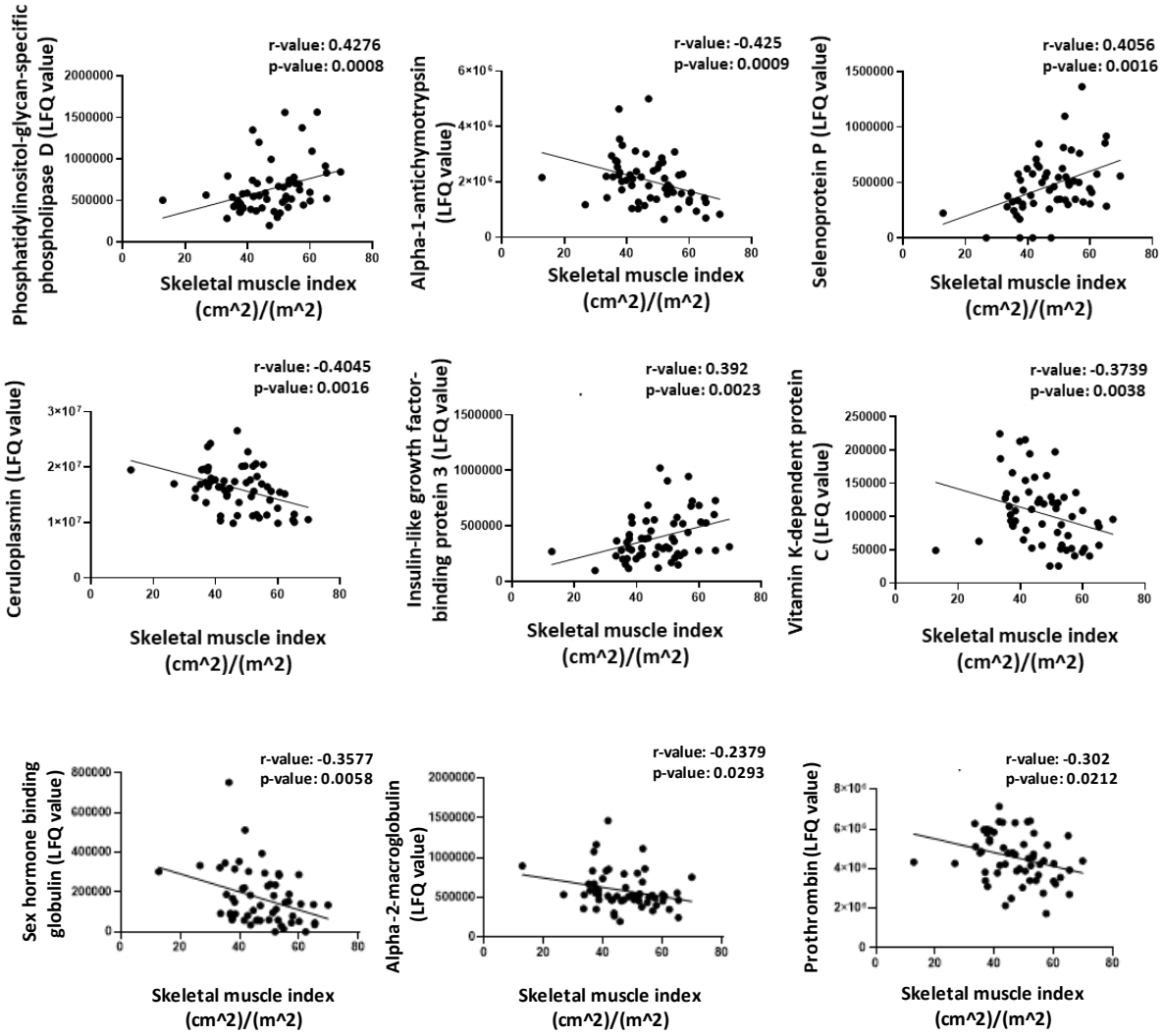
Protein	AUC
Sex hormone binding globulin	0.652
Immunoglobulin lamda variable 1-8	0.701
78kDA glucose-regulated protein	0.737
Vitamin K-dependent protein C	0.668
Ceruloplasmin	0.690
Prothrombin	0.700
Alpha-2-macroglobulin	0.680
Alpha-1-antichymotrypsin	0.712
Thyroxine-binding globulin	0.714
Selenoprotein P	0.653
Insulin growth factor protein 3	0.655
Apolipoprotein L1	0.684
Apolipoprotein A2	0.696
Phosphatidylinositol-glycan-specific phospholipase D	0.689
Beta-Ala-His dipeptidase	0.637
SUN domain-containing protein 3	0.649

AUC of proteins significantly affected by sarcopenia. AUC was determined by ROC analysis in SPSS.

(A)


r-value: -0.6336
p-value: <0.0001
Skeletal muscle index (cm^2)/(m^2)
80
60
40
20
-30
-20
-10
0
10
20
Sarcopenia score

(B)


Phosphatidylinositol-glycan-specific phospholipase D (LFQ value)
r-value: 0.4276
p-value: 0.0008
Skeletal muscle index (cm^2)/(m^2)
Alpha-1-antichymotrypsin (LFQ value)
r-value: -0.425
p-value: 0.0009
Skeletal muscle index (cm^2)/(m^2)
Selenoprotein P (LFQ value)
r-value: 0.4056
p-value: 0.0016
Skeletal muscle index (cm^2)/(m^2)
Ceruloplasmin (LFQ value)
r-value: -0.4045
p-value: 0.0016
Skeletal muscle index (cm^2)/(m^2)
Insulin-like growth factor-binding protein 3 (LFQ value)
r-value: 0.392
p-value: 0.0023
Skeletal muscle index (cm^2)/(m^2)
Vitamin K-dependent protein C (LFQ value)
r-value: -0.3739
p-value: 0.0038
Skeletal muscle index (cm^2)/(m^2)
Sex hormone binding globulin (LFQ value)
r-value: -0.3577
p-value: 0.0058
Skeletal muscle index (cm^2)/(m^2)
Alpha-2-macroglobulin (LFQ value)
r-value: -0.2379
p-value: 0.0293
Skeletal muscle index (cm^2)/(m^2)
Prothrombin (LFQ value)
r-value: -0.302
p-value: 0.0212
Skeletal muscle index (cm^2)/(m^2)

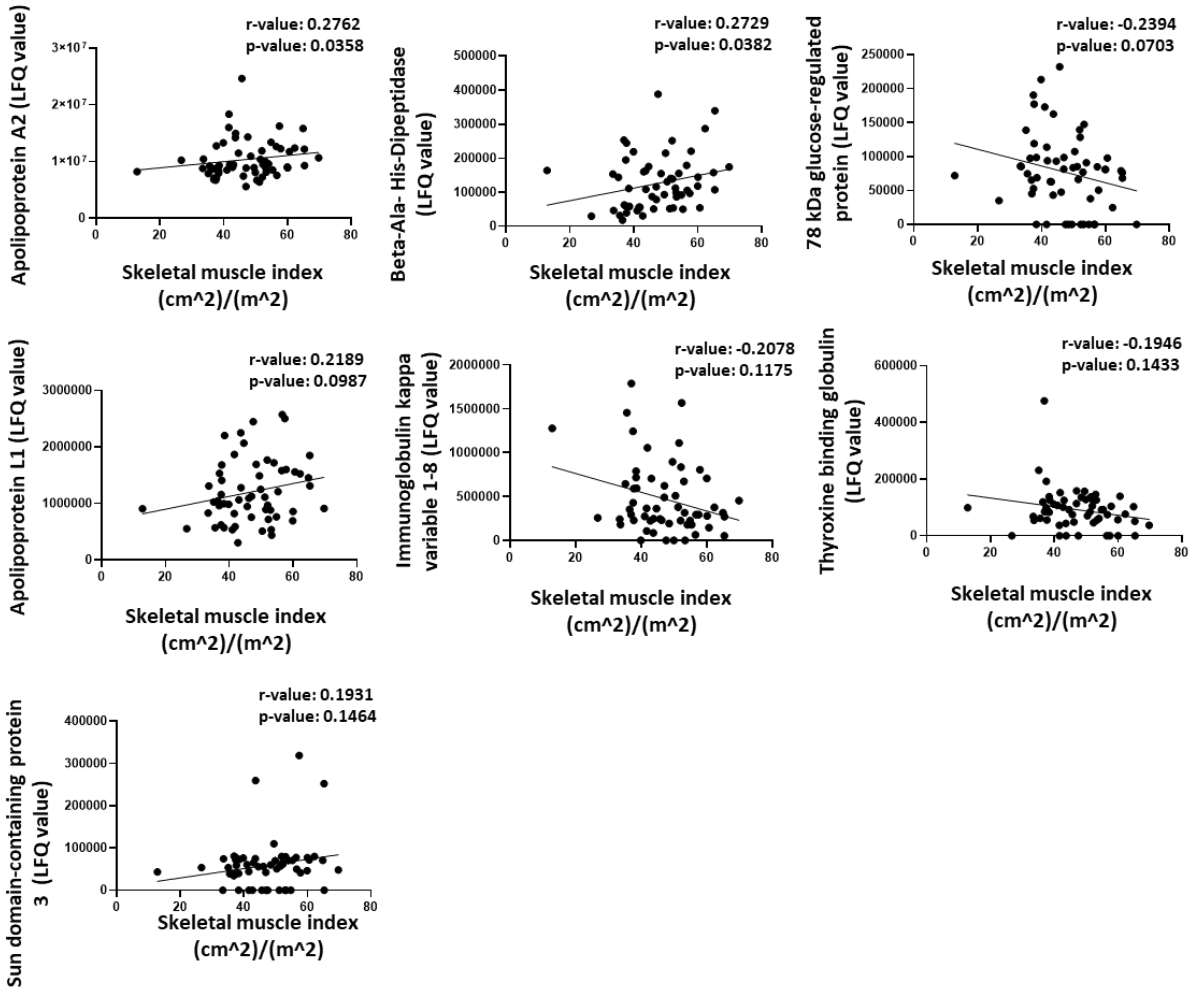


Figure 5: (A) XY dot plot demonstrating the correlation between the sarcopenia score and SMI. (B) Proteins that were significantly affected by sarcopenia and their correlation with SMI. Correlation analysis was conducted in GraphPad Prism using Spearman correlation or Pearson correlation in the case of normally distributed data.

Discussion

This study has demonstrated for the first time that specific changes within the HDL proteome may be harnessed to screen for patients with significant wasting during cancer treatment. Importantly the HDL proteome signature reflected weight loss more effectively than handgrip-strength and common biochemistry based identifiers of cachexia (albumin, CRP). Our findings also highlight the complex pathophysiology underpinning cachexia with the collation of several biomarkers into risk-scores, providing greater insights into cachexia and/or sarcopenia status, than any individual biomarker alone. While statistics were not carried out due to the low *n* number, patients misdiagnosed as having cachexia or sarcopenia using our novel risk-scores but did not meet the weight-based metrics for cachexia or SMI-based metrics for sarcopenia, exhibited significant modulation within their HDL proteome. This was coupled with higher BMI, adipose tissue, CRP and neutrophils but lower SMI, indicative that our novel biomarker panels may identify high-risk patients at early stages of wasting. Our findings collectively indicate that our novel biomarker panels may be utilized as early and sensitive indicators of cachexia and sarcopenia, enabling timely intervention to improve morbidity and survival.

Moving beyond cachexia diagnosis being solely reliant on weight-based metrics including accurate weight recall by the patient, CT scans for muscle quantification and staff to complete the assessment is critical. Often by the time cachexia is diagnosed, the patient has already lost a significant proportion of their lean body mass. Earlier detection of the highest-risk patients to enable preventative approaches to preserve body mass is of utmost importance to improve survival. There are currently no blood-based biomarkers utilized in the clinic despite several proteins being identified in the literature such as CRP, albumin, tumour necrosis factor-α (TNFα) and IL-6. The clinical utility of these proteins is challenged by their lack of specificity. This is particularly the case with CRP[133]. CRP is reportedly elevated in cancer cachexia, however as an acute phase protein it lacks specificity and is often used as an indicator of infection[469] and is also increased in the elderly[470] and people with obesity[471, 472]. Albumin is often decreased in numerous studies involving cachexia patients[199] and is considered a biomarker of prognosis[473]. However, much like CRP, it lacks specificity and has been indicated in several disease types[474]. Additionally, with a half-life of 3 weeks[475], albumin may not accurately reflect the current status of the patient, making it a less reliable marker during rapid progression. Within our study, CRP and albumin did not significantly differ between our patients with cachexia and controls and in turn the experimental mGPS score which incorporates these blood-based biomarker performed extremely poorly (AUC of 0.536 which is indicative of no prognostic value). The GLIM score, which has also been investigated for its clinical utility, relies on both weight/muscle measurements and an etiologic feature such as reduced food intake or inflammation[476]. While this

score performed well in our cohort with (AUC=0.904) it faces the same challenges as the traditional cachexia diagnostic criteria, such as accurate weight recall and skeletal muscle quantification. These findings again highlight the urgent need for novel biomarkers of cachexia that move beyond weight-based metrics and CRP, which represents a very non-specific pan inflammatory marker.

This study has shown for the first time that the HDL proteome is sensitive to the presence of cachexia and sarcopenia and has the potential to act as a novel biomarker in patients with gastrointestinal cancer. We demonstrate that the HDL proteome of CC patients is enriched with Ig Kappa chain C region (IGKC), thyroxine binding globulin (SERPINA7), alpha-1-antichymotrypsin (SERPINA3), ceruloplasmin (CP) and vitamin K-dependent protein C (PROC) and depleted in vitronectin (VTN), beta-ala-his dipeptidase (CNDP1), phosphatidylinositol-glycan-specific phospholipase D (GPLD1), ApoA2, HGFAC, ApoB, ApoL1 and SUN domain-containing protein 3 (SUN3). When these proteins, were combined into a scoring algorithm, with a cut-off value of 0.96, 70.2% of patients with cachexia were correctly identified. This score-based approach performed better by ROC AUC than any one single protein, validating a multi-protein approach.

Notably, SERPINA3 and CP belong to the acute phase response family indicative that the HDL proteome is reflecting an elevated inflammatory state in patients with CC[147]. The acute phase response (APR) has been indicated in both patient and mouse models of cachexia[335, 477]. APPs are largely produced in the liver in response to inflammatory cytokines such as IL-6-, IL-1β-, TNFα- and INFγ-like cytokines[478, 479]. In humans, IL-6, has been identified as a driver of cachexia[445, 446], is thought to be the main regulator of APP production in the liver[479]. Additionally, some studies have shown that amino acids derived from skeletal muscle proteolysis are used by the liver for APP production[68, 183, 447]. While not extensively studied, the APR may not only be a marker of cachexia but drive its development by increasing resting energy expenditure, as was found in patients with pancreatic cancer[3, 179]. SERPINA3, or alpha-1-antichymotrypsin, is a serine protease inhibitor produced as a secretory protein in the liver in response to IL-6[479]. SERPINA3 helps to maintain homeostasis by binding to proteases such as cathepsin g and chymotrypsin, but it has also been implicated in lipid metabolism, wound healing and the innate immune system[480]. Increased SERPINA3 levels in tumours have been identified as a potential biomarker of aggressive cancer and worse prognosis in several cancer types including colon cancer[481, 482]. Studies show this effect may be mediated through enhanced tumour cell migration and metastasis[483], increased proliferation[482] and immune suppression of the tumour microenvironment[484]. SERPINA3n, a mouse form of SERPINA3, has also previously been identified as a biomarker of cachexia in the muscle, serum and liver of the C26 mouse model of cachexia and correlated with body weight[485]. This result was replicated by Massart et al., in which SERPINA3n was upregulated in the muscle of C26

tumour-bearing mice compared to controls while SERPINA3n mRNA was also upregulated in BaF3 and KP53 cachectic mice[335]. This study also showed SERPINA3 was upregulated in the muscle of patients with cancer compared to healthy controls, however, it was not upregulated in cachectic vs not cachectic patients[335]. Despite this, SERPINA3 levels in muscle negatively correlate with SMI and positively correlate with CRP[335]. This mimics our results in which HDL-associated SERPINA3 also correlates with SMI (r=-0.2316,p=0.034) and CRP (r=0.2840,p=0.0102) (Supplementary Figure 2), albeit weakly. SERPINA3 had the greatest prognostic ability of any single protein (AUC=0.72) within the cachexia score (overall AUC=0.810).

CP is the major copper-carrying protein in the blood and is produced in the liver[486]. It has antioxidant capacity mediated through ferroxidase activity, converting toxic ferrous iron to ferric iron[487], but has also been identified as a pro-oxidant molecule via its ability to oxidise LDL[488, 489]. HDL becomes enriched with CP and other APPs during the APR[227] converting HDL from anti-inflammatory and anti-oxidant to pro-inflammatory and pro-oxidant[490, 491]. Increased serum CP has previously been associated with coronary heart disease[309], obesity[492], metabolic syndrome[307] and type II diabetes[308, 493]. While it has not previously been implicated in cachexia, increased CP has been identified as a biomarker of several cancer types including oral[494] and cervical cancer[495] as well as a marker of survival[496-498]. A Finnish longitudinal study conducted over 8 years identified elevated serum ceruloplasmin as a predictor of cancer occurrence and was particularly associated with lung cancer in men[499]. As an individual biomarker HDL-associated CP exhibited an AUC of 0.681 in its ability to differentiate between people with and without cachexia and an AUC of 0.690 in its ability to differentiate between people with and without sarcopenia.

SERPINA7 was identified as a predictor of both cachexia (AUC=0.690) and sarcopenia (AUC=0.714). It is produced in the liver and carries the majority of the thyroid hormones thyroxine and triiodothyronine (T3) in the blood[500, 501]. There has been very little research into the role of thyroid hormones in cachexia. However, one study of cancer patients receiving palliative care found that T3 predicted lean tissue mass[502] while another older study reported significantly higher T3 levels in patients with cachexia compared to those without[503]. In comparison to our results, this study found no difference in SERPINA7 (in serum) between cachectic and non-cachectic patients (24.9 ± 3.7 µg/ml and 25.5 ± 7.0 µg/ml respectively)[503]. Alternatively, another study found no difference in several thyroid hormones between cancer patients and controls[504]. Elevated serum SERPINA7 has been associated with hepatocellular carcinoma in a number of historic studies[505-507], while more recently it has been identified as a biomarker of chronic obstructive pulmonary disease (COPD)[501], a disease which also experiences a high rate of cachexia[508].

ApoL1 is downregulated in patients with cachexia and sarcopenia and had an AUC of 0.712 and 0.684 respectively, making it the second most prognostic protein identified within the cachexia biomarker panel. ApoL1 is an HDL-associated protein that plays a role in lipid transport, the innate immune system[509] and insulin-related metabolism[510]. Indeed high-BMI is a determinant of ApoL1 levels in non-diabetic individuals[510], and thus the reduction in ApoL1 within our study may be a confounder of excessive weight-loss in the CC group compared to the C-C group. ApoL1 is upregulated in several cancer types and has shown great diagnostic and prognostic potential, often in conjunction with other proteins in the form of a risk score, in pancreatic cancer[511-515], clear cell renal cell carcinoma (ccRCC)[516, 517], breast cancer[518], bladder cancer[519, 520], hepatocellular carcinoma[521] and melanoma[522], among others. ApoL1 has been identified as an oncogene by promoting proliferation through NOTCH1 signalling in pancreatic cancer[511] and also a tumour suppressor gene, where overexpression reportedly prevented metastasis in ccRCC[523]. ApoL1 production is stimulated by TNFα[524], which has been implicated in the development of cachexia[180, 525], making the reduction seen in our study surprising. This decrease could be due to increased utilisation of the protein, reducing its availability in circulation. ApoL1 is also an autophagy related protein[524], with increased expression inducing autophagy mediated cell death in both cancerous and non-cancerous cells[526-528]. Autophagy has been implicated, albeit controversially, in the development of cachexia[529-533] and so may be linked to the reduction of ApoL1 seen in our study. Interestingly, autophagy was increased in the livers of C26 tumour bearing mice with cachexia[534]. As the liver is the largest producer of ApoL1[535], this heightened autophagy could contribute to the decrease seen on the HDL in our study.

Collation of proteins into a cachexia risk-score within our study resulted in a good ability to differentiate between patients with and without cachexia within a relatively small sample size (AUC=0.810; sensitivity=0.702 and specificity=0.115). These promising results highlight that a panel of biomarkers may provide greater precision in identifying patients at highest risk of developing cachexia. Interestingly, a number of patients appear to have been 'misdiagnosed' with cachexia who do not fit the criteria based on their weight-based metrics. This 'misdiagnosed' group exhibited significantly higher CRP and neutrophil counts than the correctly diagnosed C-C patients, indicative that the HDL proteomics score may identify patients at early stages of cachexia prior to significant weight-loss, and in particular those with elevated levels of inflammation. Similarly, there were a number of patients within our study who met the criteria for a diagnosis of sarcopenia (men: if BMI <24.99 than a SMI of 43 cm^2/m^2 is used, if BMI >25 than a SMI of <53 cm^2/m^2 is used, women: <41 cm^2/m^2 for all BMI) who did not fulfil the criteria for cachexia based on their self-reported weight-loss over the prior 6 months. These individuals exhibited a similarly elevated 'cachexia score' relative to patients with both cachexia alone and cachexia + sarcopenia indicative that the risk-score can identify wasting phenotypes

independent of weight-loss. It will be of great interest in the future to identify the prognostic capacity of this risk-score at predicting who will develop cachexia within a larger study cohort to validate these findings.

Within a secondary analysis of our data, we sought to identify whether a biomarker panel could be developed to identify sarcopenia. Accurate identification of sarcopenia is extremely challenging and limited to costly or time-consuming assessments such as handgrip strength or gait speed[536, 537 36]and diagnostic imaging[538]. Within this study we identified enrichment of HDL with 78 kDa glucose-regulated protein (HSPA5), Prothrombin (F2), Alpha-2-macroglobulin (A2M), Sex hormone-binding globulin (SHBG), Vitamin-K dependent protein C (PROC), Immunoglobulin Kappa Variable 1-8 (IGKV1-8) and SERPINA7 and depletion of Beta-Ala-His dipeptidase (CNDP1), Apolipoprotein A-II (ApoA2), Phosphatidylinositol-glycan-specific phospholipase D (GPLD1), Apolipoprotein L1 (ApoL1), Insulin-like growth factor-binding protein 3 (IGFBP3), Selenoprotein P (SEPP) and SUN domain-containing protein 3 (SUN3) in the setting of sarcopenia. Collation of these proteins into a score had an AUC of 0.861, sensitivity of 0.813 and specificity of 0.154. This score preformed better than any single protein included within the score and correctly identified 81.25% of patients with sarcopenia. While the scores were not significantly different between the C-S and C+S groups (supplementary figure 2), we believe further refinement and investigation may allow us to differentiate between the two cachexia subtypes, due to subtle differences in the identified proteins.

The proteins A2M and F2, which were included in the sarcopenia score and individually correlated with SMI (r= -0.2379 and -0.3020 respectively), also belong to the APR[147]. F2 is produced in the liver and is involved in coagulation and thrombosis[539]. After vascular injury, it is converted into its active form thrombin which has many roles including aiding in clot formation, monocyte migration and platelet activation[540]. While increased thrombin generation has been identified in several cancers[541] and a C26 mouse model of cachexia[542], its precursor prothrombin has not been extensively studied in the cancer space. Patients with cancer are at an increased risk of thrombosis, linked to greater thrombin activation in response to the tumour and chemotherapy[543]. Further research would be needed to determine if HDL-associated prothrombin correlates with active thrombin in our cohort.

A2M is an APP with the ability to inhibit a wide range of proteases[544]. It has several additional functions including immune cell chemotaxis and modulation, and acts as a carrier protein for cytokines and damaged extracellular proteins[544]. While A2M has been reported as both increased and decreased in cancer, it was upregulated in the skeletal muscle of patients with cachexia[545] and in the liver of cachectic C26 mice[542]. A study on HDL sub-species found that HDL enriched with A2M lost its protective

effects and was associated with an increased risk of coronary heart disease[546]. This would suggest that the HDL of patients with sarcopenia in our cohort has lost its cardioprotective properties. A2M binds to many proteins in serum including cytokines[544]. While the impact of cytokine-A2M binding on the functional properties of the cytokines is poorly understood, it is known that both IL-8 and IL-6, which are implicated in cachexia and sarcopenia[119, 200, 240, 547], become resistant to protease degradation [548-550]. Therefore, it is plausible that increased A2M may facilitate increased cytokine availability, and drive muscle atrophy, which would be interesting to explore in the future.

HSPA5 is a heat-shock family member that typically resides in the endoplasmic reticulum (ER)[551] and acts as a chaperone in the unfolded protein response (UPR)[552]. HSPA5 helps to initiate the UPR by detaching from the transmembrane proteins, Protein kinase RNA-activated-like ER Kinase (PERK) , Activating Transcription Factor 6 (ATF6) and Inositol Requiring Enzyme 1α (IRE1α) to trigger several events that help to alleviate ER stress[553, 554] such as protein synthesis inhibition (PERK mediated)[555], and increased production of chaperone proteins such as HSPA5 (ATF6 mediated)[556, 557]. However, during chronic ER stress, such as during inflammatory conditions, these proteins can contribute to cell death via apoptosis and autophagy activation[558-560]. The UPR has been tentatively implicated in the development of mouse models of cachexia[558]. Bohnert et al., determined that the UPR is upregulated in the muscle of two mouse models of cancer cachexia, including increased expression of HSPA5 in the muscle of ApcMin/+ cachectic mice[561]. They determined that this was potentially a compensatory mechanism in response to stress and found that blocking the UPR worsened muscle wasting and muscle strength in both cachexia models and control mice[561]. HSPA5 has also been identified as a pro-tumourigenic marker in several cancers[562-564]. Increased ER stress causes HSPA5 to translocate to the cell surface where it can aid in pro-tumourigenic signalling by promoting treatment resistance[565], proliferation and apoptosis suppression[566, 567] and metastasis[568]. As the stage and metastasis occurrence are relatively similar between our groups, the increase in HSPA5 in the sarcopenic group is unlikely to be caused by more aggressive cancer types and so this increase may be a result of sarcopenia or a direct contributor to sarcopenia development.

While biomarker discovery to date has widely focused on serum/plasma as the matrix for novel signals of cachexia[61]; our study has specifically focused on investigating HDL-specific proteins to overcome the limitations of serum as a matrix including over-representation of more abundant proteins and the large dynamic range of proteins present[569]. HDL particles are derived predominantly from the liver, carry a plethora of pro- and anti-inflammatory proteins as well as metabolic proteins[311], providing greater insights into a person's immune-metabolic status[313, 570]. Additionally, the liver is increasingly being explored as a potential contributor to cancer cachexia[100, 101, 460, 462] and so it is not surprising that the

liver-derived HDL can act as a cachexia biomarker. Indeed, many of the proteins identified in this study, such as the APPs are predominantly made in the liver[147] and are subsequently enriched on circulating HDL particles.

A strength of this study is the well-matched nature and clinical characterisation of the cancer cachexia and cancer control groups, minimising confounding variables. There are several important limitations – firstly all patients are at different stages of treatment upon sample collection - it is thus not possible to discern cancer-specific cachexia protein signatures from treatment-induced cachexia signatures. It is however noteworthy that most patients were between their 1st-3rd treatment cycle, in both cachexia and no cachexia groups, ensuring that treatment-induced effects on the HDL proteome are controlled for. Future analysis of patient samples prior to commencement of chemotherapy would be key to unravel disease-driven cachexia from treatment-induced cachexia. Secondly, this study was exploratory by nature utilizing a discovery proteomics approach to identify potential biomarkers of cachexia. The resulting biomarker panels will need to be validated in larger patient cohorts using more direct measurements of the specific HDL-associated proteins (e.g. utilizing multiplex ELISA or targeted mass spectrometry approaches). Thirdly, the current study is cross-sectional by nature with outcome data not available to discern the potential of the biomarker panel to predict outcomes in affected patients. It would be of great interest in the future to determine the prognostic capacity of these biomarker panels to predict both the extent of cachexia (even prior to significant weight-loss) as well as overall survival. Finally, our study focused on GI-related cancers. It will be of great interest to cross compare cachexia in multiple different cancer types to discern whether the cachexia biomarkers identified are applicable to multiple different types of cancer, or specific for GI-related cancers.

In conclusion, we have shown that the HDL proteome can reflect distinct pathophysiological changes caused by cachexia and sarcopenia in a gastrointestinal cancer cohort. We demonstrate enrichment of HDL particles with different acute phase proteins including SERPINA3 and CP in the setting of cachexia, and A2M and F2 in the setting of sarcopenia. Finally, we demonstrate that collation of significantly altered proteins into scoring algorithms can be harnessed to diagnose both cachexia and sarcopenia and outperforms any given individual protein in prognostic capacity. Our findings highlight that more complex biomarker panels will ultimately be required to sensitively identify and diagnose patients with, or at high-risk of developing, cancer cachexia.

Supplementary data

Supplementary Table 1: Treatment plan and chemo cycle number of C-C and CC patients.

		Cancer without cachexia (n=26)	Cancer with cachexia (n=47)	p-value
Treatment plan	Chemotherapy	30.80%	21.30%	
	Chemotherapy and surgery	53.80%	36.20%	
	ChemoRadiation and surgery	7.70%	21.30%	
	Definitive ChemoRadiation	7.70%	21.30%	ns
	1	19.23%	31.91%	
	2	30.77%	25.53%	
	3	11.54%	17.02%	
	4	11.54%	8.51%	
	5	4.26%	4.26%	
	6	3.85%	0.00%	
	7	0.00%	2.13%	
	8	0.00%	2.13%	
Chemo Cycle	14	7.69%	0.00%	ns

Supplementary Table 2: Treatment plan and chemo cycle number of C-C, C-S and C+S patients.

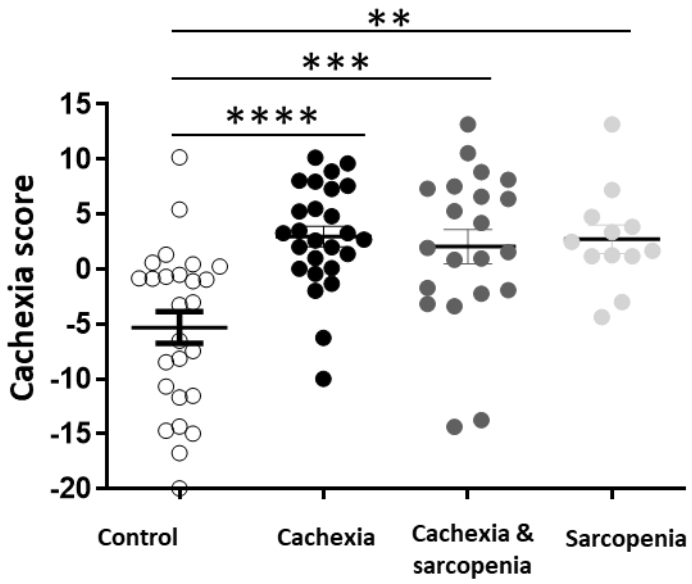
		Cancer without cachexia (n=26)*a	Cancer cachexia without sarcopenia (n=26)*b	Cancer cachexia with sarcopenia (n=21)*c	p-value
Treatment plan	Chemotherapy	30.80%	23.10%	19.00%	ns
	Chemotherapy and surgery	53.80%	34.60%	38.10%	
	ChemoRadiation and surgery	7.70%	19.20%	23.80%	
	Definitive ChemoRadiation	7.70%	23.10%	19.00%	
Chemo Cycle	1	19.23%	26.92%	38.10%	
	2	30.77%	26.92%	23.81%	
	3	11.54%	19.23%	14.29%	
	4	11.54%	15.38%	0.00%	
	5	4.26%	3.85%	4.76%	
	6	3.85%	0.00%	0.00%	
	7	0.00%	0.00%	4.76%	
	8	0.00%	0.00%	4.76%	
	14	7.69%	0.00%	0.00%	ns

Supplementary Table 3: Phenotypic features of C-C patients that were misdiagnosed and not misdiagnosed by the cachexia score

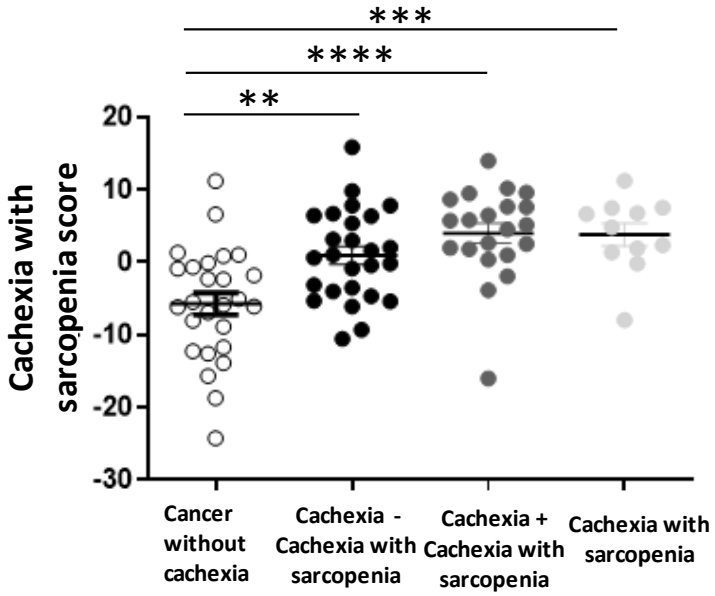
	Not misdiagnosed as having cachexia (*n*=23)	Misdiagnosed as having cachexia (*n*=3)
Age (years)	62.97 ± 1.73	64.76 ± 9.78
Height (meters)	1.68 ± 0.02	1.67 ± 0.11
Weight at survey (kg)	77.97 ± 3.09	85.77 ± 7.37
Pre diagnosis weight (kg)	80.8 ± 3.38	85.9 ± 10.27
6 months weight change (%)	0.37 ± 0.73	3.21 ± 3.3
Body Mass Index at survey	27.46 ± 1.03	31.03 ± 3.23
Total adipose tissue area (cm^2)	349.38 ± 41.56	503.43 ± 157.5
Skeletal Muscle Index (cm^2)/(m^2)	54.94 ± 1.68	51.86 ± 2.52
Hand grip (kg)	27.1 ± 2.05	21.17 ± 2.74
C-reactive protein (mg/L)	9.39 ± 3.6	45 ± 40.07
Albumin (g/L)	34.7 ± 0.75	32.67 ± 2.19
Lymphocytes (cell count x10^9/L)	1.33 ± 0.16	1.57 ± 0.6
Neutrophils (cell count x10^9/L)	3.64 ± 0.36	5.17 ± 1.19
Neutrophil to lymphocyte ratio	3.66 ± 0.59	4.32 ± 1.35
White cell count (cell count x10^9/L)	5.7 ± 0.52	7.73 ± 2.08
Haemoglobin (g/dl)	11.93 ± 0.31	11.77 ± 0.92

Supplementary Table 4: Phenotypic features of C-C patients that were misdiagnosed and not misdiagnosed by the sarcopenia score

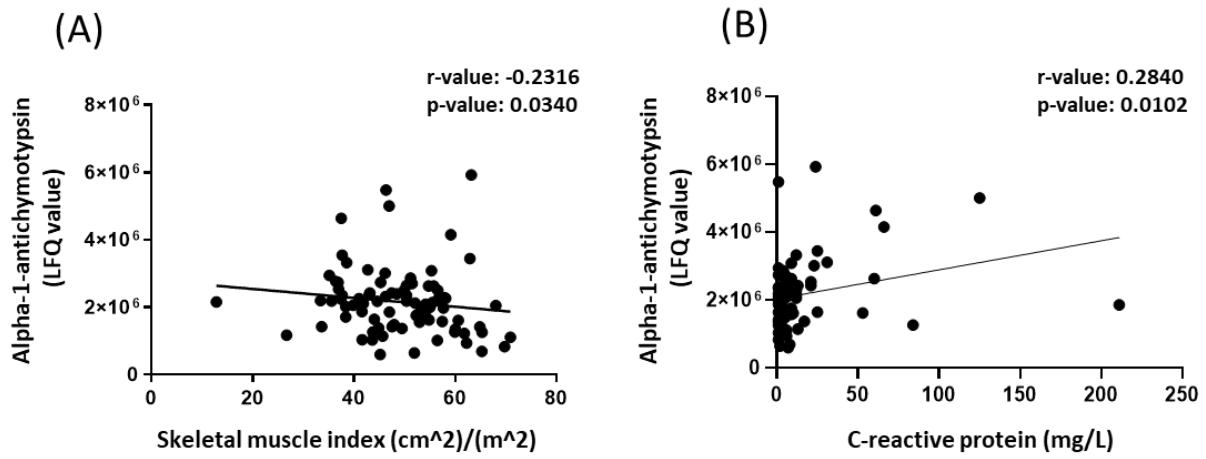
	Not misdiagnosed as having sarcopenia (n=22)	Misdiagnosed as having sarcopenia (n=4)
Age (years)	62.86 ± 1.81	64.95 ± 6.92
Height (meters)	1.68 ± 0.02	1.7 ± 0.08
Weight at survey (kg)	78.1 ± 3.23	83.08 ± 5.86
Pre diagnosis weight (kg)	81.17 ± 3.52	82.6 ± 7.98
6 months weight change (%)	0.29 ± 0.76	2.91 ± 2.35
Body Mass Index at survey	27.64 ± 1.06	29.13 ± 2.98
Total adipose tissue area (cm^2)	348.8 ± 43.59	467.94 ± 116.89
Skeletal Muscle Index (cm^2)/(m^2)	55.34 ± 1.71	50.44 ± 2.28
Hand grip (kg)	27.22 ± 2.14	22.03 ± 2.12
C-reactive protein (mg/L)	8.77 ± 3.71	39.5 ± 28.86
Albumin (g/L)	34.91 ± 0.76	32 ± 1.68
Lymphocytes (cell count x10^9/L)	1.25 ± 0.14	1.95 ± 0.57
Neutrophils (cell count x10^9/L)	3.43 ± 0.3	5.88 ± 1.1
Neutrophil to lymphocyte ratio	3.71 ± 0.62	3.89 ± 1.05
White cell count (cell count x10^9/L)	5.36 ± 0.42	8.93 ± 1.89
Hemoglobin (g/dl)	12.04 ± 0.3	11.28 ± 0.81



Supplementary figure 1: Dot plot of cachexia score-subgroup illustration. Cachexia score of the Sarcopenia group which was not included in the original analysis**.** Significance was determined by Kruskal Wallis analysis in GraphPad prism. p****<0.0001,p***<0.001,p**<0.01,p*<0.05



Supplementary figure 2: Dot plot of sarcopenia score-subgroup illustration. Sarcopenia score of the C-C, C-S, C+S and S groups. Significance was determined by Kruskal Wallis analysis in GraphPad prism. p****<0.0001,p***<0.001.



Supplementary figure 3: Correlation of alpha-1-antitrypsin (LFQ) with (A) Skeletal muscle index and (B) CRP. Correlation analysis was carried out in GraphPad prism

Supplementary Table 5: Average LFQ of all proteins identified on the HDL of C-C and CC.

Gene names	C-C	CC
SERPINA1	1030823	1383436
IGLV8-61	140523.5	153637.2
IGLV3-16	327813.5	395138.5
IGLV3-10	3218665	2552140
IGKV2D-29	4584145	5373662
IGHV1OR15-1	2368561	2176955
ITIH3	1268839	1314534
IGKV2-40	2069123	2517695
SAA2-SAA4	2892012	3229727
F5	234266.5	190060.3
IGKV3D-11	1829227	1874583
IGHG1	54406000	55494489
IGHD	421144.1	762129.9
IGHV3-49	1213556	1449087
C1QB	191753.2	169730.1
IGHV3-15	1055407	919214.9
IGHV3-73	190723	258680.5
IGHV3-74	272278.3	349584
IGLL5	18649454	16105889
SHBG	134566.7	166271.8
IGKV6-21	212782.7	251600.6
IGHV1-18	2867021	2383113
IGHV4-28	1328929	1772831
IGHV3-38	814751.5	767929.2
IGHV5-51	3080408	2936019
IGHV2-70	957887.3	1007270
IGKV1-8	301178.6	465394.3
IGKV1-6	433610	577110

IGHA2	2555219	1856702
C4A	3652785	3916962
IGLV5-45	208994.3	149041.6
IGHV3-30	6216127	5529391
PROS1	597925.4	551277.2
SEPP1	544735.8	450691.1
IGHG3	34512746	34438894
IGHG2	21209846	20599623
IGHG4	12659142	14502466
CFHR2	1932985	1670021
C1R	369166.2	332915.1
IGHV3-72	14401562	13417079
LDHB	138727.9	104859.2
IGLC7	629564.2	999403.1
SERPING1	8530115	8204781
AGT	191160.2	220500.7
HSPA5	51869.23	75091.4
A0A8Q3SI05	230298.6	230318.2
C9	1128253	1240750
C5	8629785	8322402
C1QC	535331.2	467863.6
C8A	1444230	1357770
A0A8Q3WL56	306864.6	304296.7
IGFBP3	463306.5	366420.4
APOC3	8779969	7164872
CFB	4637581	4810613
GC	451746.5	488360.4
PROC	89333	110110.1
CFI	714875.8	741781.7
CLEC3B	1047155	964354.7
C2	453944.2	523223.8
TFRC	250887.1	235446.6
SERPINA10	272849.2	256624.7
FCGR3A	281093.4	320193
KLKB1	3325600	3092207
SUN3	79305.27	43352.11
APOC4-APOC2	1087233	935168.1
APOC1	681543.8	622012.3
QSOX1	164010.1	189457.6
APOL1	1421880	986821.1
CD5L	30529.88	35499.87
ATRN	678692.3	628861.6
APOM	1198782	1217024
CP	14840927	16945404
F2	4098719	4606626
HP	14818888	16653029
HPR	225751	345054.1

F9	231280.5	230180.6
F10	705195.8	599325.5
PLG	131098.6	156197.2
SERPINC1	677772.7	658471.9
SERPINA3	1770684	2383160
A2M	515597.3	592430
C3	39815500	39154149
KNG1	4879669	4511830
IGJ	364563.5	311641.3
IGKV1-5	1617138	1606790
IGKV3-20	1679698	1787748
IGKV3-15	1927393	1668855
IGLV1-47	2651881	2392894
IGLV1-51	205612.3	192698.2
IGLV3-19	135518.2	218878.7
IGHV1-69	4284490	4641154
IGHV3-7	8719519	7572677
IGKC	49446808	63231149
IGHM	1580325	1346816
IGHA1	30626538	30722451
APOA1	36885962	34184723
APOE	2814339	2359016
APOA2	11199946	9217313
FGA	172547.6	175338
APCS	1766854	1701455
APOH	397964.3	307294
FN1	394986.7	271790.9
AMBP	7499946	6264289
AHSG	1211246	1341783
TF	2301558	2534362
HPX	7389915	8365309
C4BPA	447007.3	540517.4
VTN	5312519	4810664
CAT	80674.23	167788
APOB	383597.7	314523.6
LCAT	214676.2	205736
HRG	531152.3	464614.3
A1BG	1890919	1959557
VWF	208516.4	204249.8
APOD	6290896	5542228
F13B	969058.5	902468.3
SERPINA7	65372.92	118312
SERPIND1	2490027	2484747
	860658.8	909932.8
IGKV4-1	3019372	2993629
IGHV4-61	5732285	6638734
APOA4	1726372	1693164

C8B	116019.2	102013.2
C8G	748017.3	808731.5
CFH	1153486	1050991
SERPINF2	1622345	1642773
DBH	80132.15	80665.87
C1S	3954350	4031987
C4A	13554419	13905268
C4B	990550.4	1196257
P0DOY3	46244962	45468894
P0DTE1	130231.3	127339.9
C7	3174373	2959104
CLU	6489673	5840230
C6	2769754	2544389
SELL	328871.5	350067.2
CPN1	148858.5	127148.8
IGLL1	101473.5	123393
ORM2	340830.4	324968.1
ITIH2	6609119	5772381
ITIH1	2150933	1795551
GPX3	235746.1	230792.1
CPN2	169008.8	176999
PROZ	86479.96	88815.15
	879882.4	484162.9
FBLN1	535959.2	453626.2
PON1	3728662	3805168
CFP	198567.7	162809.6
CDH5	67173.08	67139.98
IGFALS	719371.5	709688.3
BTD	141928.2	139621.6
AFM	1798755	1570128
MASP1	331881.5	268724.8
LUM	3047454	2849151
CDH13	102700.2	89032.85
ACTB	312072.7	316715.9
HBB	1880291	2947349
HBA1	2307226	3145035
GPLD1	720057.7	574234.3
CFHR1	3000123	3151320
HGFAC	616900.8	472448.5
EFEMP1	306682.3	298442.3
APOF	145867	171120.4
ITIH4	9883612	9720574
PON3	80205.75	75499.81
TGFBI	448951.5	447545.5
ECM1	134307.5	124035.7
VASN	99000.08	96921.91
CNDP1	145125	105756.9

PGLYRP2	206787.2	235375.7
C1RL	829375	676935.5
SMC3	1191213	1533240

Chapter 4:

HDL Proteome Remodelling and Hepatic Alterations in a Mouse Model of Cancer Cachexia

HDL Proteome Remodelling and Hepatic Alterations in a Mouse Model of Cancer Cachexia

Rianna McElroy[1], Morgane M. Thibaut[2], Laure B. Bindels[2], Fiona McGillicuddy[3#], Helen M. Roche[1,4*]

1. School of Public Health, Physiotherapy and Sport Science, UCD Conway Institute, UCD Institute of Food and Health, University College Dublin (UCD), Dublin, Ireland.

2. Metabolism and Nutrition Research Group, Louvain Drug Research Institute, UCLouvain, Université Catholique de Louvain, Brussels, Belgium,

3. Diabetes Complications Research Centre, UCD Conway Institute and School of Medicine, University College Dublin, Dublin 4, Republic of Ireland.

4. Institute for Global Food Security, Queen's University Belfast, Belfast, UK.

***Corresponding Author 1:** Professor Helen M. Roche

Address: UCD Conway Institute, University College Dublin, Belfield, Dublin D04 H1W8, Ireland

Email: helen.roche@ucd.ie

Telephone: +353 1 716 6845

#Corresponding Author 2: Assistant Professor Fiona C. McGillicuddy

Address: UCD Conway Institute, University College Dublin, Belfield, Dublin D04 H1W8, Ireland

Email: fiona.mcgillicuddy@ucd.ie

Telephone: +353 1 716 6769

Chapter prepared as manuscript, journal to be decided.

Abstract

Introduction: Cancer cachexia is a prevalent and yet underdiagnosed condition that affects many cancer patients. It results in weight loss, reduced appetite, shortened survival and poor quality of life. Screening for this disease is hindered by a lack of a consensus definition, poor awareness, and the costly and time consuming nature of many of the assessments, such as CT scans for muscle quantification. Therefore, a circulating biomarker would greatly improve the diagnosis of cancer patients. We propose the High Density Lipoprotein (HDL) proteome as a novel biomarker for the detection of this disease. Using a C26 mouse model of cachexia, we explored differences between the HDL proteome of mice with cachexia (C26), mice with cancer but not cachexia (NC) and control mice (CT). This allowed us to support previous research in a human cohort of gastrointestinal cancer patients and additionally, explore the relationship between the liver derived HDL proteome and changes in the liver. This study also allowed us to identify hepatic pathways that are significantly altered by cachexia.

Methods: C26, NC and CT mice were provided by Prof. Laure Bindels. HDL was isolated from the plasma of mice using size exclusion chromatography and further purified using a lipid removal assay (LRA), before trypsin digestion. Proteomics was also performed on liver and gastrocnemius tissue. Tissue was pulverised on dry ice before protein extraction and peptide digestion using the PreOmics iST kit. Peptides were analysed on a timsTOF mass-spectrometry and searched in MaxQuant against the mouse database. T-tests were carried out in Perseus to determine significant proteins ($p<0.05$ for HDL and FDR for tissues). Ingenuity pathway analysis (IPA) was carried out on liver and gastrocnemius tissue to identify pathways.

Results: The HDL proteome is significantly different from CT and NC mice (*n*=46 and *n*=72 significant proteins respectively) while there were fewer differences between CT and NC mice (*n*=16). The HDL of C26 mice was enriched with SAA1,SAA2,SAA4, and A2Mp and depleted of HGFAC, C9 and PROC when compared to CT. The HDL of C26 mice was enriched with ApoB, SAA4, TF, CP and HP, and depleted of SPP2, LAP3, CDH13, HGFAC and IGFBP3 when compared to NC mice. We provided further evidence to support our human biomarkers of cachexia and sarcopenia (low muscle mass) including ApoB, CP, HGFAC, PROC, SERPINA3n, SERPINA7, VTN, ApoA2 and IGFPB3. The livers of C26 where significantly different from CT and NC mice, while there was no difference between the CT and NC mice (*n*=366 and *n*=690 respectively). The most significant pathways include those related to protein synthesis and xenobiotic metabolism.

Conclusion: We have shown, in a mouse model, that the HDL proteome can act as a biomarker of cachexia, validating prior work in a human cohort. We have also shown that the liver is greatly affected by cachexia, with increased protein synthesis and decreased xenobiotic metabolism.

Introduction

Cancer cachexia is "a multifactorial syndrome defined by an ongoing loss of skeletal muscle mass (with or without loss of fat mass) that cannot be fully reversed by conventional nutritional support"[2]. It affects up to 80% of cancer patients, with highest prevalence in pancreatic, gastrointestinal and lung cancer[1, 571]. This disease leads to functional impairment[572] with reduced appetite[573], poor quality of life[574], treatment tolerance[440] and survival[575, 576]. Despite its prevalence, cachexia is largely undiagnosed in the clinical setting[577, 578] as screening for cachexia is not part of routine care[58]. This is partly due to the lack of treatment options[33], however, other major issues include lack of a consensus definition, expensive and time consuming assessments such as CT scans for skeletal muscle quantification and adequate staff to complete assessment[55, 579]. Identifying reliable blood based biomarkers that can be integrated into routine care would greatly improve early diagnosis, monitoring and treatment outcomes for patients. While the literature has identified a number of candidate biomarkers for cachexia, to date none have been approved for clinical use.

Typical biomarkers of cancer cachexia can be characterised as inflammatory, muscle or fat wasting products, mRNA or hormones. Tumour necrosis factor-alpha (TNF-α), also known as cachectin, was one of the first inflammatory cytokines identified in the development of cachexia[580, 581]. It is known to induce anorexia[582], activate the ubiquitin proteasome pathway (UPP) through nuclear factor kappa B (NF-κB)[583-586], reduce adipocyte lipoprotein lipase activity[587-589], and induce lipolysis[587, 590] and the acute phase response[335]. While it has been proposed as a biomarker of cachexia in several studies and cancer types[67-69], it has not been consistent[108, 447], likely due to the its short half-life (18.2 mins)[71], low bioavailability[71] and low reproducibility[73]. Interleukin-6 (IL-6) is a promising potential biomarker of cachexia that has been identified in various cancer types[134, 173, 176, 445, 446, 591-594]. It is elevated in early and late stages of disease[96] and promotes cachexia through white adipose tissue browning[96], lipolysis[96, 445], the UPP[595, 596] and autophagy[175, 595]. While IL-6 appears to be a robust cachexia biomarker candidate, it is also upregulated in obesity[185, 597, 598], infection[599-601] rheumatoid arthritis[602, 603] and cardiovascular disease[604], making it non-specific. Muscle and adipose tissue wasting or catabolism could also yield biomarkers of cachexia. Amino acids for example are consistently decreased in cachexia[605, 606], as they are shuttled to the liver for gluconeogenesis[607] and acute phase protein production[462] and to the tumour for growth and proliferation[608]. Cala et al., found a decrease in several amino acids and their derivatives with arginine, tryptophan, indoleacetic acid and threonine particularly affected[605]. More et al., demonstrated *n*=13 amino acids, including leucine, isoleucine, arginine and tryptophan, were reduced in a cohort of patients with gastrointestinal, liver and ovarian cancer[606]. Miller et al., developed a model that included *n*=6 metabolites that identified cachexia[609]. This model included *n*=2 amino acids, L-proline and L-phenylalanine, *n*=2 fatty acids, hexadecanoic acid

and octadecenoic acid and *n*=2 lysophospholipids Lyso-PC 16:1 and Lyso-PC 18:2[609]. Despite some promising advancements, there are challenges that may impede the development of metabolomics as a reliable biomarker source. These include the influence of diet, lifestyle, microbiome and potentially the tumour and also the need for fasted patient samples, in a cohort at risk of weight loss.

As cachexia is a complex multi-organ[610] inflammatory[60] and metabolic[611] response to cancer, a biomarker panel that incorporate multiple proteins reflecting both inflammatory and metabolic processes may provide a more accurate and robust method of disease identification, some of which outperform individual biomarkers[515, 612]. To this end, we have previously established two biomarker scores in gastrointestinal cancer patients that can identify cachexia or sarcopenia (low muscle mass) using the High Density Lipoprotein (HDL) proteome (Chapter 3). HDL particles are liver derived (primarily)[613], protein-rich lipoproteins[614] primarily known for their well-established role in cholesterol homeostasis[615] and cardiovascular disease (CVD) risk[615]. While initial research focused on circulating HDL levels in CVD, current research have revealed more extensive biological implications relating to the complex HDL proteome and related functionality[311, 616, 617]. The HDL proteome is composed of ~285 proteins[223] including those related to inflammation (serum amyloid A (SAA), ceruloplasmin (CP) and alpha-1-antichymotrypsin (SERPINA3))[618], and oxidation (serum paraoxonase 1)[619]. Under normal conditions, HDL mediates cholesterol efflux and possess anti-inflammatory, anti-oxidative and anti-thrombotic properties[618]. However, during inflammatory conditions, the HDL can become enriched with pro-inflammatory proteins such as SAA and depleted of beneficial proteins such as ApoA1 which can affect these functions[354, 355, 618]. Additionally, several studies have shown that the HDL proteome is altered during disease states such as cardiovascular disease[226, 298, 620-622] and diabetes[224, 623, 624] but also during obesity[376, 570, 625] and aging[362]. This metabolic rearrangement of the HDL proteome can yield distinct proteomic signatures that may have biomarker potential[298, 626].

The HDL proteome holds particular promise for cancer cachexia, as it is a comprehensive cargo particle for not only cholesterol and phospholipids but also several inflammatory and cellular stress response proteins[60, 627, 628]. The majority of HDL is also formed by the liver[629], an organ that is gaining attention for its potential role in cachexia[610]. Although research on the liver's involvement in cachexia is still in its early stages, several emerging trends are becoming evident in the literature including alterations in lipid metabolism[630-632] and mitochondria[633, 634], infiltration of immune cells[635-637] and upregulation of protein synthesis[638, 639], gluconeogenesis[463] and inflammation[637]. Liver lipid metabolism is disrupted in various cachexia models by reductions in fatty acid oxidation[632, 640-643]. This decrease was attributed to the low levels of carnitine seen in some models of cachexia[640, 642], and reduced mRNA expression and activity of carnitine palmitoyl-transferase I (CPT-I) and CPT-II[640-643]. CPT-I and -II are essential for

shuttling fatty acids across the mitochondrial membrane[644] and disruptions in their activity lead to accumulation of fat in the liver known as steatosis[640]. Also contributing to the development of steatosis in cachexia is the downregulated production and secretion of Very Low Density Lipoprotein (VLDL) in the cachectic liver[631, 640, 641]. In the Walker 256 rat model of cachexia, this is due to reductions in the expression of microsomal triglyceride transfer protein (MTP) and apolipoprotein B (ApoB)[640, 641], while in the C26 mouse model, it is associated with upregulation of transcription factor transforming growth factor beta 1-stimulated clone 22 D4 (TSC22D4)[631], which correlated with levels of tissue wasting[631].

Inflammation is a key feature in cancer cachexia, with cytokines released by the tumour and host immune cells driving many of its pathways[60, 628]. In the liver, inflammation may be responsible for many of the changes observed, for example TNF-α and IL-1β reduce the hepatic expression of MTP[640, 645], while inflammation impairs the hepatobiliary system, causing cholestasis in mice[100]. Inflammatory cytokines, particularly IL-6, also lead to induction of the acute phase response in the liver[479, 646, 647], a hallmark of cancer cachexia[3]. The acute phase response forms part of the innate immune system and is activated in response to injury in an effort to restore homeostasis[648, 649]. However, during chronic inflammation, like cachexia, the continuous activation of this system may lead to increased resting energy expenditure, promoting a wasting phenotype[179]. The acute phase response may be further driven in cachexia by the release of amino acids from wasting muscle which are used by the liver for the production of acute phase proteins[462, 650]. CRP, a positive acute phase protein[147] and albumin, a negative acute phase protein[147] are frequently cited, albeit inconsistently[111, 134, 135], as biomarkers of cachexia[651, 652]. The modified Glasgow prognostic score (mGPS), a scoring system based on CRP and albumin levels, has been proposed as an objective diagnostic score for cancer cachexia[159]. However, while it has shown potential as a prognostic score, correlating well with overall survival[467], its association with cachexia and skeletal muscle mass is not as consistent[134, 653-655].

HDL is greatly affected by the acute phase response, with reduced circulating levels[656, 657], as seen in cachexia[111, 658, 659], and significant remodelling of the HDL proteome[660, 661]. This remodelling includes the incorporation of acute-phase proteins[660], most famously SAA[353, 355], which impact HDL's functional properties and result in the development of pro-inflammatory HDL if unresolved[227]. Building on this understanding, our previous study in cancer patients investigated the biomarker potential of the HDL proteome for the identification of cachexia and sarcopenia. We found that the HDL of patients with cachexia or sarcopenia was enriched with acute phase proteins such as CP, SERPINA3 and alpha-2-macroglobulin (A2M) and depleted of apolipoproteins such as apolipoprotein L1 (ApoL1) and apolipoprotein A-II. Using these HDL proteins, we created cachexia and sarcopenia scores, each of which had excellent performance (Chapter 3).

The aim of the current study was to support our previously determined biomarker signature in humans, using the C26 mouse model of cachexia (C26) (Kindly provided by Prof. Laure Bindels from the Université Catholique de Louvain, Belgium) by comparing the HDL of C26 mice to a non-cancer causing model of colon cancer (NC), and control mice without cancer (CT). Recognizing the crucial role of the liver in cancer cachexia and the interaction between the acute phase response and HDL composition, we also sought to explore the connection between HDL composition and liver using proteomics. In this study, we have shown for the first time that the HDL proteome is significantly affected by the presence of cancer cachexia and is enriched with acute phase proteins such as SAA2, SAA4, CP and SERPINA3n in mice. We have also shown that there is some overlap with the proteins significantly affected in the liver and on the HDL proteome, indicating that the HDL proteome may be sensitive to hepatic changes. Finally, we have shown that while there are significantly more changes to the HDL proteome of cachectic mice, there were some interesting similarities to our work in cancer patients. This work illustrates a novel area of research in the cancer cachexia space and highlights the utility of the HDL proteome for biomarker development.

Methods:

Animals:

Mouse plasma, liver and gastrocnemius muscle were kindly provided by Professor Laure Bindels from the Université Catholique de Louvain, Belgium. A detailed account of the mice is available in the original publication[662]. Cachexia was induced using a well known cachexia-causing colon carcinoma 26 (C26) model which is characterised by both muscle and adipose tissue loss[663-666]. A non-cachexia causing C26 colon carcinoma (NC) (TKG0518; Resource Centre for Biomedical Research, Tohoku University) model was also used for comparison. Male CD2F1 mice (7 weeks old, Charles River Laboratories, Italy) were kept on a 12hr light/dark cycle in pathogen free, individually ventilated cages and fed an irradiated chow diet (AO4-10, Safe, Augy, France). After 7 days acclimatisation, mice were injected subcutaneously in the upper flank with 1 × 106 cells in 0.1 mL saline of cultured C26 or NC cells or saline as a control (CT). Mice were anesthetised (isoflurane gas, Abbott, Wavre, Belgium) 10 days post inoculation after a 6hr fast and harvested tissue frozen in liquid nitrogen[662]. Tissue transferred to the University College Dublin for this study was kept on dry ice before being stored at -80 ◦C.

HDL and LDL isolation:

Plasma was mixed with 0.01 M phosphate buffer saline (PBS) (Sigma-Aldrich) and separated using x2 sequential superose 6 Increase 10/300 GL columns (Cytiva) linked to a GE ÄktaPure FPLC system (Cytiva). The resulting chromatogram allows for identification of fractions containing the VLDL, LDL, HDL and serum peaks (resulting chromatograms in Figure 7). LDL fractions (Fr) chosen include Fr 18-21 for CT and as LDL-Peak 1 for NC and C26, LDL-peak 2 for NC and C26 include Fr 22-25. HDL fractions chosen include Fr 32-36 for CT, NC and C26-HDL peak 1 while C26-HDL peak 2 include FR 35-38.

HDL proteomics preparation:

HDL fractions were pooled and enriched for lipid-containing particles by incubation with lipid removal agent (100mg/ml). The resulting pellet was washed twice in 50mM Ammonium bicarbonate (ABC) and then resuspended in 2M urea made up in 50mM ABC. 5mM of Dithiothreitol (DTT) was added to samples and incubated for 30 minutes at 60°C. 10mM Iodoacetamide (IAA) was added and incubated in the dark at room temperature for 30 minutes. Samples were diluted with 50µl of ABC to reduce urea to a working range for the trypsin. 0.5µg of trypsin, using Trypsin singles (Sigma-Aldrich), was added to each sample and incubated overnight at 37°C with shaking. Formic acid (1% final concentration) was added to inhibit the trypsin and centrifuged for 5min at 10,000 RPM. Supernatant containing peptides was transferred to a fresh tube and peptides were loaded onto C18 Ziptips (ThermoFisher scientific) and eluted in buffer containing 70% acetonitrile and 0.1% formic acid. Samples were dried down in a Concentrator 5301 (Eppendorf) and resuspended in 3% Acetonitrile with 0.1% formic acid.

LDL proteomics preparation:

LDL fractions were pooled and 800µl moved to a fresh tube. 20% chilled trichloroacetic acid (TCA) was added and incubated on ice for 30 min. Samples were centrifuged for 10 min (14,000 RPM) at 4 ◦C and the supernatant removed. The resulting pellet was washed twice with 200µl of ice-cold acetone (centrifugation 2 min 14,000 RPM). The pellet was air dried and resuspended in 2M urea made up in 50mM ABC. 5mM of Dithiothreitol (DTT) was added to samples and incubated for 30 minutes at 60°C. 10mM Iodoacetamide (IAA) was added and incubated in the dark at room temperature for 30 minutes. Samples were diluted with 50µl of ABC to reduce urea to a working range for the trypsin. 0.5µg of trypsin, using Trypsin singles (Sigma-Aldrich), was added to each sample and incubated overnight at 37°C with shaking. Formic acid (1% final concentration) was added to inhibit the trypsin and centrifuged for 5min at 10,000 RPM. Supernatant containing peptides was transferred to a fresh tube and peptides were loaded onto C18 Ziptips (ThermoFisher scientific) and eluted in buffer containing 70% acetonitrile and 0.1% formic acid. Samples were dried down in a Concentrator 5301 (Eppendorf) and resuspended in 3% Acetonitrile with 0.1% formic acid.

Liver homogenisation and digestion:

20mgs of tissue was cut on dry ice using liquid nitrogen chilled tools and weighed before 400µl of chilled LYSE buffer (PreOmics) was added. A pre-chilled metal bead was added to the Eppendorf tube and the tubes placed in pre-chilled bead-beater blocks. Tissue was homogenised in the bead-beater (Qiagen) at 3 min, 30 second frequency intervals until no particles were visible. Sample with the bead was spun for 60 seconds (1500 RCF) and supernatant transferred to a fresh tube. Centrifugation step was repeated.

Muscle homogenization and digestion:

Muscle tissue was crushed into a powder using a mortar and hammer chilled in liquid nitrogen. 20mgs of powdered muscle was weighed before adding 400µl of chilled LYSE buffer (PreOmics) and a pre-chilled metal bead. Tissue was homogenised in the bead-beater (Qiagen) at 3 min, 30 second frequency intervals until all soluble particles were in solution. Sample with bead was spun for 60 seconds (1500 RCF) and supernatant transferred to a fresh tube. Centrifugation step was repeated.

Tissue proteomics digestion:

100ng of protein from muscle and liver samples was calculated using a Pierce Bradford protein assay Kit. Protein was reduced, alkylated and digested using the PreOmics iST kit (PreOmics) as per their protocol.

Mass spectrometry on a timsTOF:

Evotips (EVOSEP) were loaded with peptides as per manufacturer's instructions. As this study was exploratory, all available peptides for the HDL and LDL proteomics were loaded onto the Evotips while the liver and muscle were normalised prior to loading. Samples were run on a timsTOF Pro mass spectrometer (Bruker Daltonics, Bremen, Germany) coupled to the EvoSep One system (EvoSep BioSystems, Odense, Denmark). Peptides were separated on a reversed-phase C18 Endurance column (15cm x 150µm ID) over 44 minutes using a flow rate of 0.5ul/min and an increasing concentration of formic acid in acetonitrile. TIMS (Trapped Ion Mobility Spectrometry) and PASEF (Parallel Accumulation Serial) modes were enabled on the timsTOF pro and operated in a positive ion polarity. Spectra was captured in the mass range from 100 to 1,700 m/z. Each PASEF cycle took 1.17 s and consisted of one MS ramp for precursor detection followed by 10 PASEF MS/MS ramps. Dr Catriona Scaife from the Conway UCD proteomic core ran the peptides from the muscle and liver samples. Dr Eugene Dillon from the Conway UCD proteomic core ran the peptides from the mouse HDL and LDL.

Proteomic data analysis:

Raw Label Free Quantification (LFQ) values were analysed in Perseus (version 1.6.15.0). Contaminants, proteins only identified by site and reverse database identifications were filtered from the dataset. A valid value filter of 70% in at least one group was applied. HDL proteins were normalised to ApoA1 Label free quantification (LFQ) prior to analysis. Proteins were LOG2 transformed and missing values imputed from the normal distribution. A paired t-test with a p-value of 0.05 was carried out comparing C26 vs CT, C26 vs NC and NC vs CT. Results were normalised using z-score and visualised as a heat map. Raw LFQ values were graphed in GraphPad prism (version 10) and significance determined using an independent t-test on normally distributed data or a Mann-Whitney U test on data that was not normally distributed.

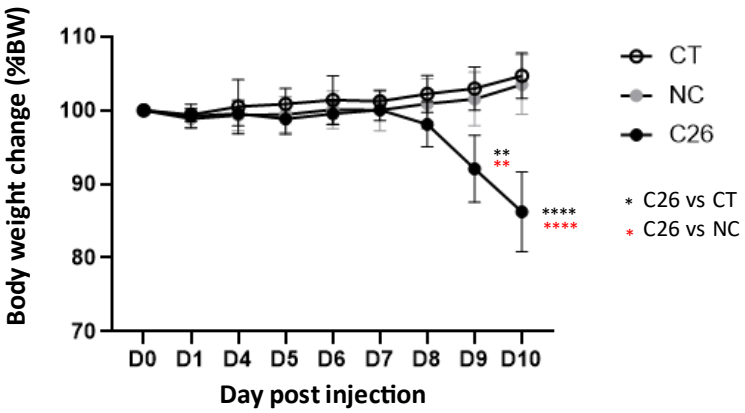
Results

Cachexia affects the bodyweight, muscle and adipose tissue of mice compared to control mice and tumour bearing mice without cachexia:

The bodyweight of C26 mice began to diverge on day 8 post implantation of tumour cells and was significantly lower from CT and NC on day 9 ($p<0.01$ and $p<0.01$, respectively) and day 10 ($p<0.0001$ and $p<0.0001$, respectively) (Figure 6A). CT and NC mice gained an average of 4.7% and 3.5% of their initial bodyweight (iBW) respectively by day 10; while C26 mice lost an average of 13.5%. NC mice had an average tumour weight of 0.9985 %iBW while the C26 tumours were much smaller at 0.546 %iBW ($p<0.05$) (Figure 6B). There was no difference in the weight of the liver among the groups.

In terms of skeletal muscle mass assessment, the gastrocnemius muscle of C26 mice was 9.1% and 17.7% smaller than CT ($p<0.01$) and NC ($p<0.001$) mice respectively on day 10 while there was no statistical difference between CT and NC mice. The tibialis muscle of C26 mice was 14.5% and 18.6% smaller than CT ($p<0.01$) and NC ($p<0.001$) mice respectively on day 10 while there was no statistical difference between CT and NC mice. The brown adipose tissue (BAT) of C26 mice was 45% and 43.5% smaller than CT ($p<0.001$) and NC ($p<0.001$) mice respectively while the subcutaneous adipose tissue (SAT)) of C26 mice was 37.2% and 43% smaller than CT ($p<0.05$) and NC ($p<0.01$) mice respectively. There was no significant difference between the BAT and SAT of CT and NC mice.

(A)



(B)

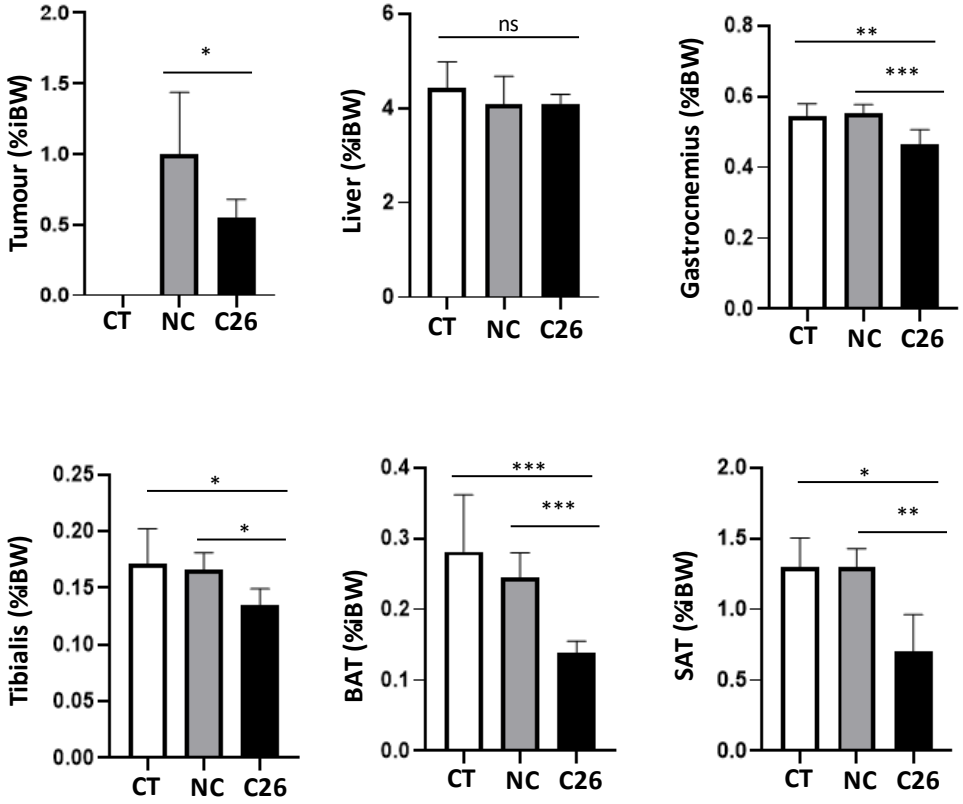


Figure 6: (A) Bodyweight evolution of CT, NC and C26 mice from day 0 to day 10, expressed as % of intial bodyweight (%iBW). (B) Weight of tumour, liver, gastrocnemius, tibialis, BAT and SAT expressed as %iBW. Analysis was carried out in GraphPad using a one-way ANOVA for normally distributed data and a Kruskal-Wallis for non-normally distributed data. **** ≤0.0001,***≤0.001,** ≤0.01,*≤0.05. All mouse work related to raising the mice, implantation of tumours, culling and recording of weights were carried out in the lab of Prof. Laure Bindels in Université Catholique de Louvain, Belgium.

Cancer affects the distibution of LDL particles and cancer cachexia affects the distribution of HDL particles in mice:

During the HDL isolation process via FPLC, a surprising result was found. The distribution of LDL particles was altered in NC and C26 mice, compared to CT mice. Also the distribution of HDL particles in C26 mice was altered, compared to CT and NC mice as shown by chromatograms in Figure 7A. Figure 7B shows a dot plot of apolipoprotein A1 (ApoA1), the characteristic HDL protein, for the pooled HDL peaks (FPLC fractions pooled to create one sample) in CT, NC and C26-HDL peak 1 and peak 2. There was no significant difference between any of the groups, indicating the presence of HDL in both C26 peak 1 and C26 peak 2. Figure 1C shows a dot plot of apolipoprotein B (ApoB), a protein strongly associated with LDL and VLDL, for the pooled LDL fractions in CT, NC-LDL peak 1, NC-LDL Peak 2, C26-LDL peak 1 and C26-LDL peak 2. ApoB was present in all groups, however there was a significant difference between CT and C26-peak 1 and NC and C26-peak 1, wherein ApoB was higher in C26-peak 1.

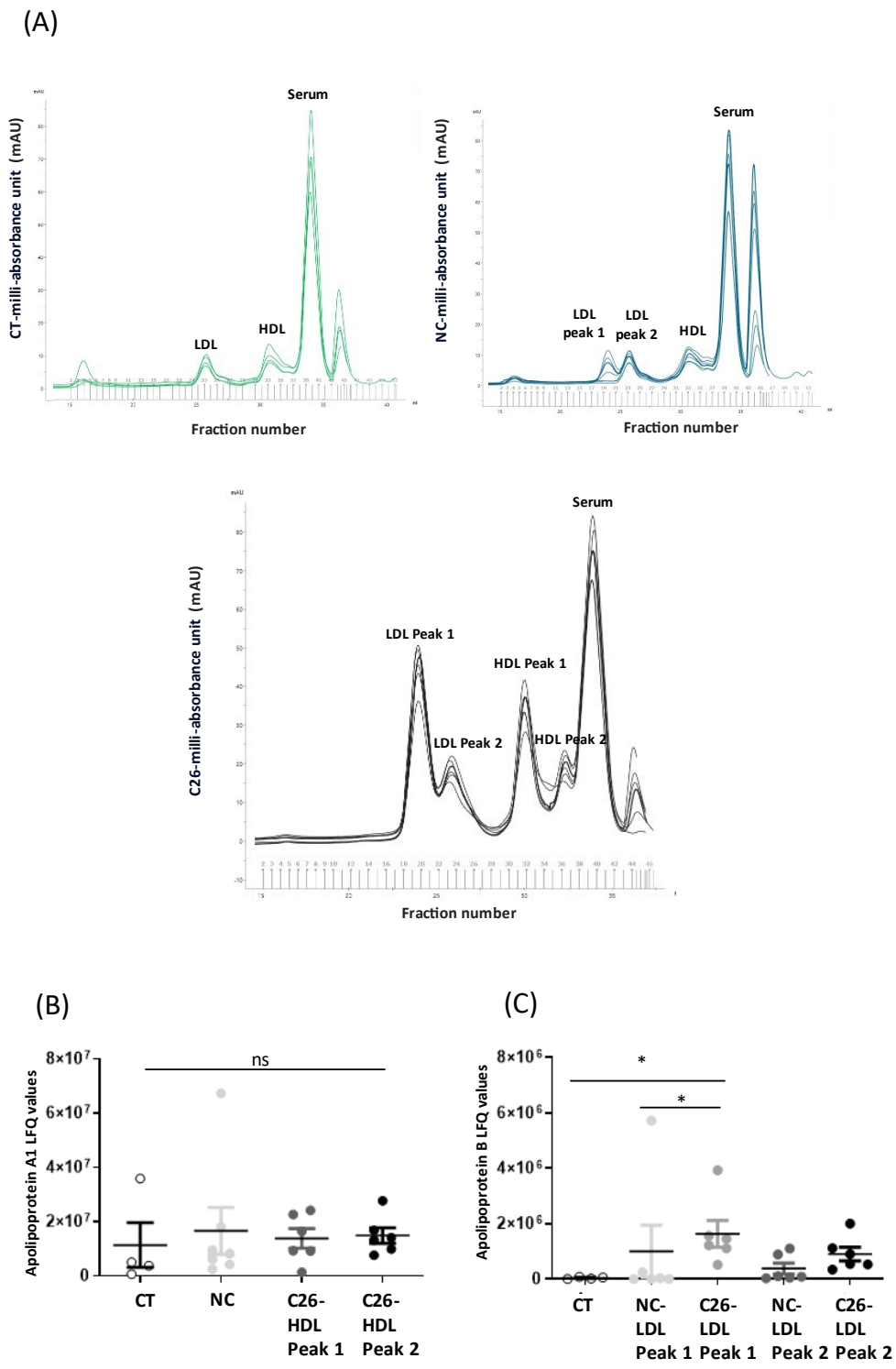


Figure 7: (A) chromatograms produced by S.E.C of plasma for CT, NC and C26 mice. Highlighted are the LDL peaks, HDL peaks and serum (B) Dot plot of ApoA1 across the HDL peaks of CT, NC, C26-HDL peak 1 and C26-HDL peak 2. (C) Dot plot of ApoB across the LDL peaks of CT, NC-LDL peak 1, NC-LDL peak 2, C26-LDL peak 1, C26-LDL peak 2. Chromatograms were produced by Cytivas Akta Pure. Chromatogram images were cropped to exclude the wash-out phase of the FPLC run for space-saving purposes. Significance of LFQ values was determined in GraphPad for display purposes. ns=not significant, p*≤0.05.

The HDL proteome is greatly affected by cancer cachexia:

To determine the effects of cancer cachexia on the HDL proteome, HDL was isolated from the plasma of CT (n=4), NC (n=6) and C26 (n=6) mice and proteomic analysis was performed. The resulting HDL proteins were normalised by ApoA1 to control for HDL abundance. Collectively, n=236 proteins were identified among all three groups, including C26-HDL peak 1 and C26-HDL peak 2, n=112 of which were shared among all groups. C26-HDL peak 2 had the greatest number of unique proteins (n=35) which included proteins such as coagulation factor VII (F7), C-reactive protein (CRP), progranulin (GRN) and ORM1, while CT had the lowest amount of unique proteins (n=3), mannose-binding protein A (MBL1) serine protease 3B (Prss3b) and Ig kappa chain V-IV region S107B (IGKV4-61) (Supplementary Table 7).

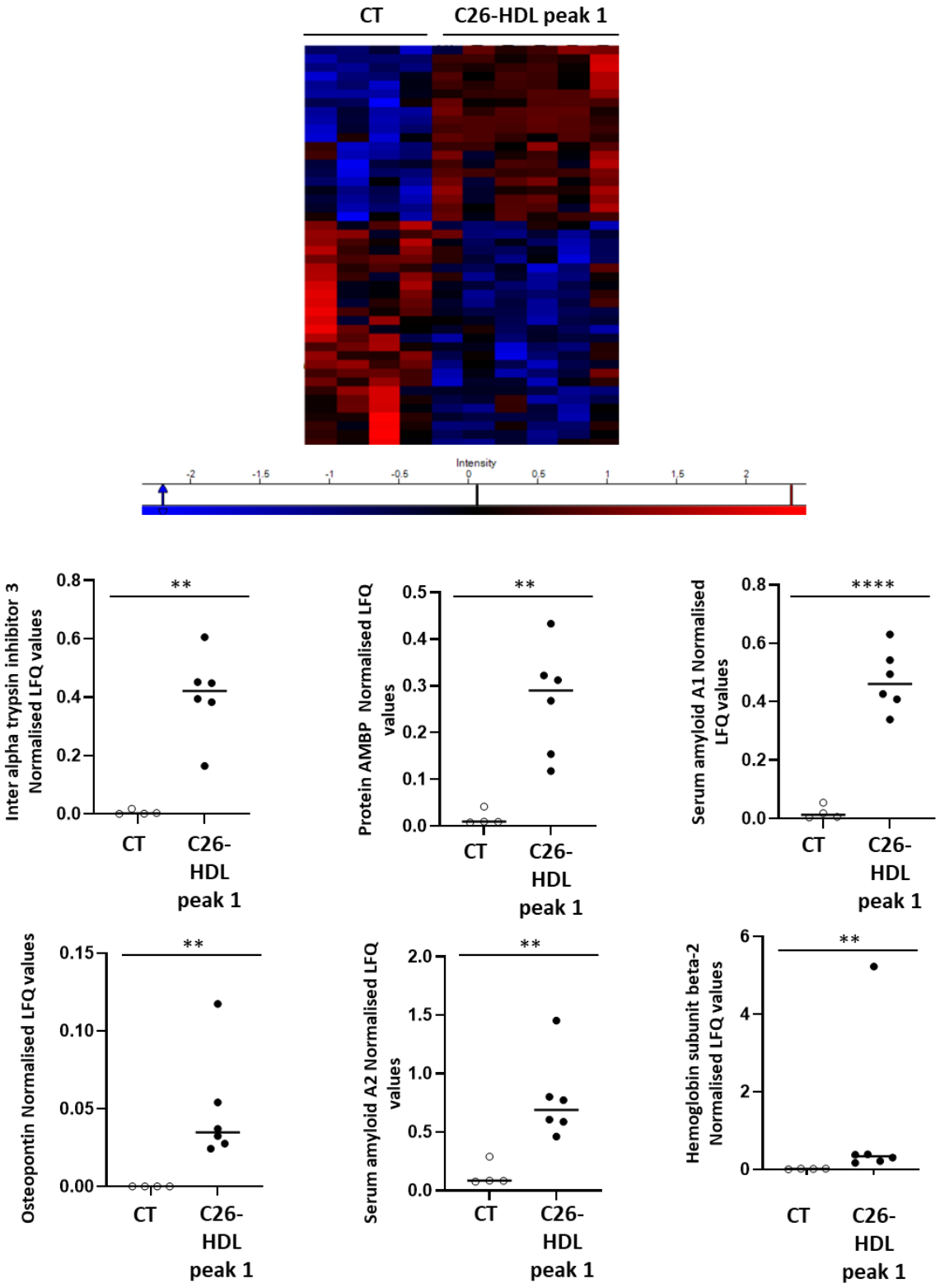
CT and NC were compared against C26-HDL peak 1, but not C26-HDL peak 2, as they were made up of the same FPLC fractions. C26-HDL peak 1 was compared against C26-HDL peak 2 to determine the make-up of this unexpected peak. When comparing CT vs C26-HDL peak 1 (Figure 8A), *n*=46 proteins were significant, *n*=20 of which were upregulated in C26-HDL peak 1 including SAA1, SAA2, serum amyloid A4 (SAA4), osteopontin (SPP1) and A2Mp, and *n*=26 of which were downregulated including hepatocyte growth factor activator (HGFAC), complement component C9 (C9), vitamin K-dependent protein C (PROC) and secreted phosphoprotein 24 (SPP2) (Full list of significant proteins in Supplementary Table 8).

When comparing NC vs C26-HDL peak 1 (Figure 8B), *n*=72 proteins were significant, *n*=35 of which were upregulated in C26-HDL peak 1 including ApoB, SAA4, TF, ceruloplasmin (CP) and HP, and *n*=37 of which were downregulated including SPP2, cytosol aminopeptidase (LAP3), CDH13, HGFAC and insulin-like growth factor-binding protein 3 (IGFBP3) (Full list of significant proteins in Supplementary Table 8).

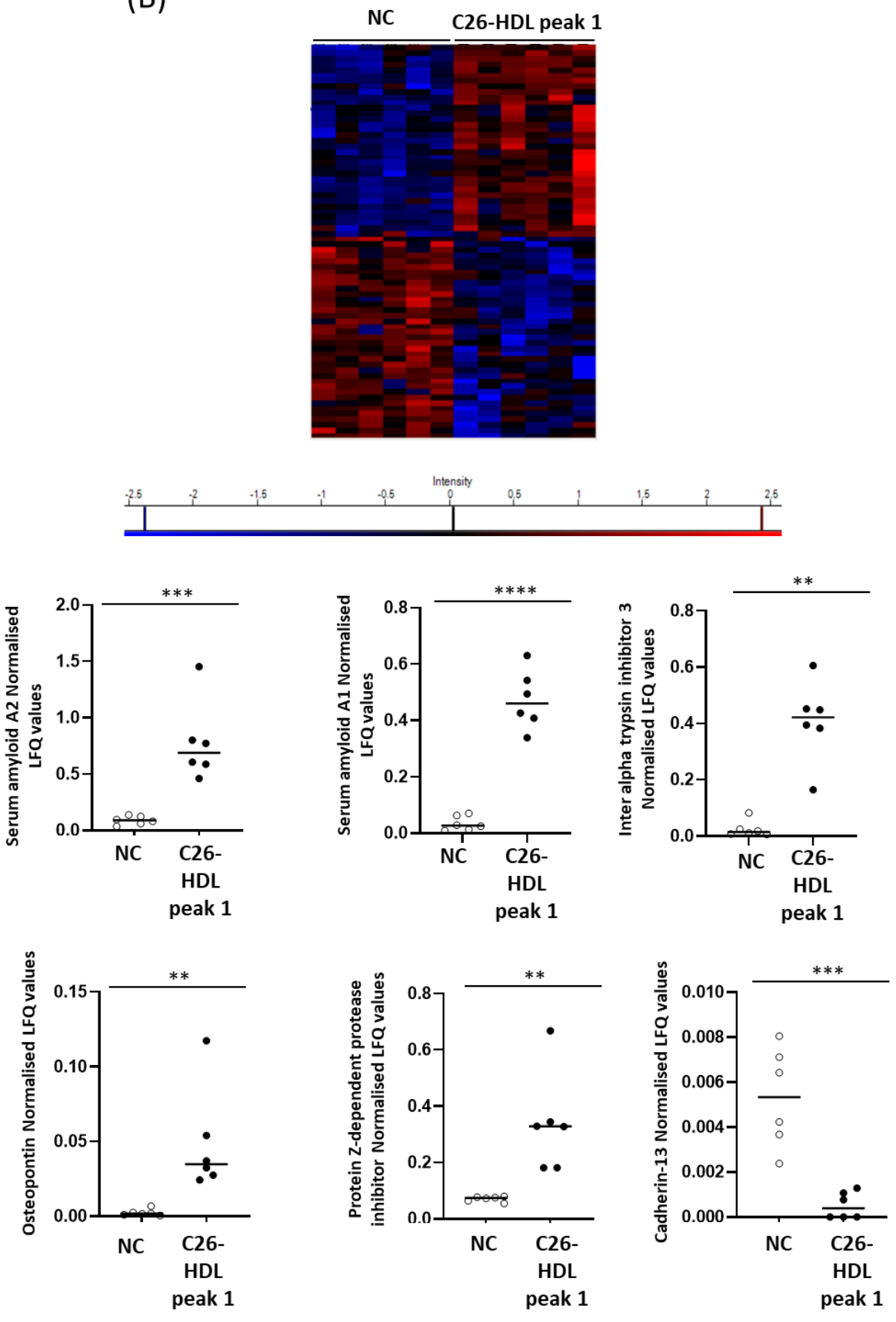
When comparing the HDL proteome of CT vs NC mice (Figure 8C), *n*=16 proteins were significantly different, *n*=8 of which were upregulated in NC including vacuolar protein sorting-associated protein 52 homolog (VPS52), HP, ITIH3, pyruvate kinase (PKLR) and mannan-binding lectin serine protease 2 (MASP2) and *n*=8 of which were downregulated including serine protease inhibitor A3K (SERPINA3K), fructose-bisphosphate aldolase (ALDOA), apolipoprotein M (ApoM) and prosaposin (PSAP) (Full list of significant proteins in Supplementary Table 8).

When comparing C26-HDL peak 1 and C26-HDL peak 2 (Figure 8D), *n*=106 proteins were significant, n=82 of which were upregulated in C26-HDL peak 2, including ICA, actin, cytoplasmic 1 (ACTB), hemopexin (HPX), gelsolin (GSN), thyroxine-binding globulin (SERPINA7), CP and IGFBP3 and *n*=24 were downregulated including SPP1, ITIH3, vasorin (VASN), apolipoprotein A2 (ApoA2) and A2M (Full list of significant proteins in Supplementary Table 8).

(A)


CT
C26-HDL peak 1
Intensity
-2
-1.5
-1
-0.5
0
0.5
1
1.5
2
Inter alpha trypsin inhibitor 3 Normalised LFQ values
Protein AMBP Normalised LFQ values
Serum amyloid A1 Normalised LFQ values
Osteopontin Normalised LFQ values
Serum amyloid A2 Normalised LFQ values
Hemoglobin subunit beta-2 Normalised LFQ values
**
****
CT
C26-HDL peak 1

(B)


NC
C26-HDL peak 1
Intensity
-2.5
-2
-1.5
-1
-0.5
0
0.5
1
1.5
2
2.5
Serum amyloid A2 Normalised LFQ values
***
2.0
1.5
1.0
0.5
0.0
NC
C26-HDL peak 1
Serum amyloid A1 Normalised LFQ values
****
0.8
0.6
0.4
0.2
0.0
NC
C26-HDL peak 1
Inter alpha trypsin inhibitor 3 Normalised LFQ values
**
0.8
0.6
0.4
0.2
0.0
NC
C26-HDL peak 1
Osteopontin Normalised LFQ values
**
0.15
0.10
0.05
0.00
NC
C26-HDL peak 1
Protein Z-dependent protease inhibitor Normalised LFQ values
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0.6
0.4
0.2
0.0
NC
C26-HDL peak 1
Cadherin-13 Normalised LFQ values
***
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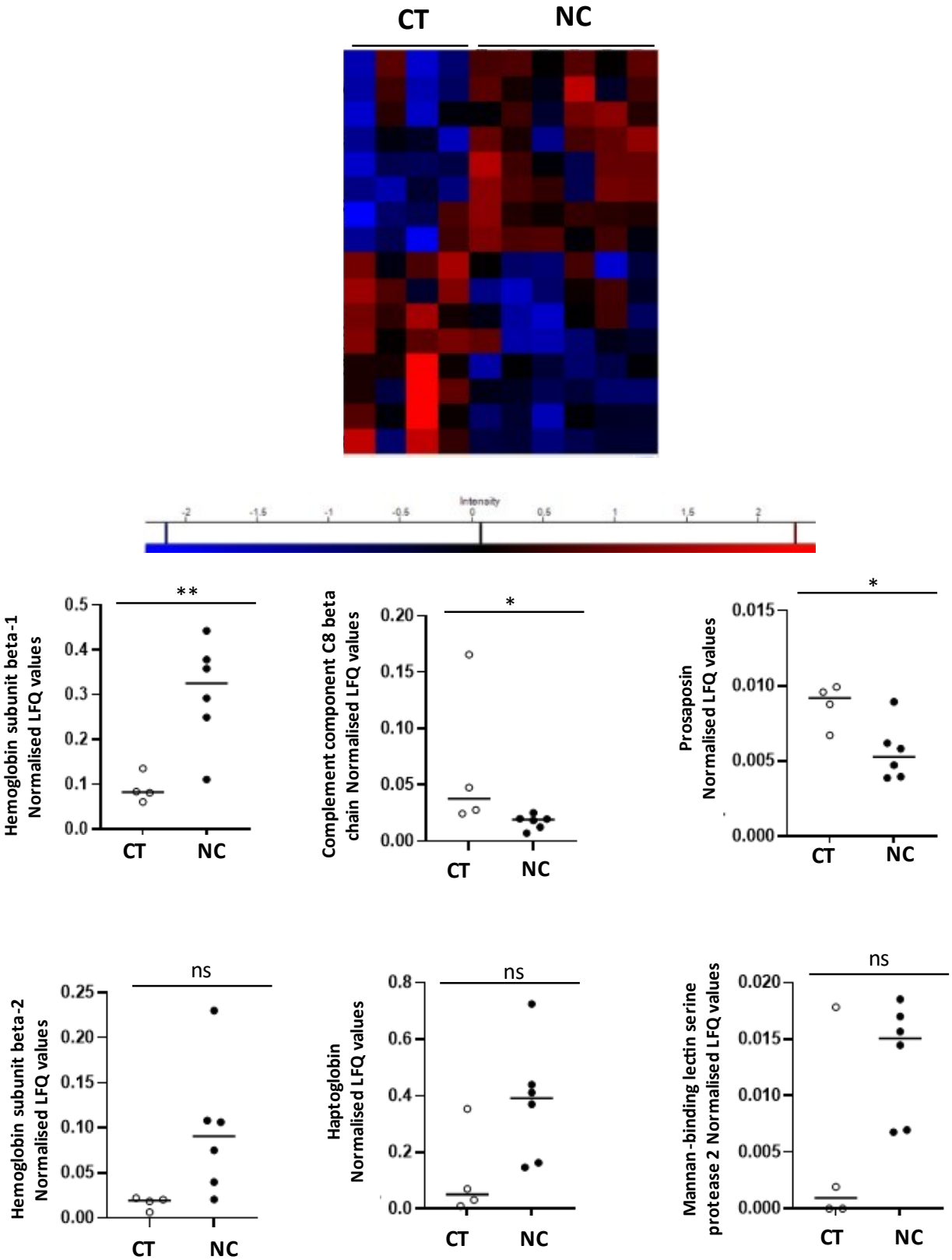
0.008
0.006
0.004
0.002
0.000
NC
C26-HDL peak 1

(C)


CT
NC
Intensity
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-1
-0.5
0
0.5
1
1.5
2
Hemoglobin subunit beta-1 Normalised LFQ values
**
0.5
0.4
0.3
0.2
0.1
0.0
CT
NC
Complement component C8 beta chain Normalised LFQ values
*
0.20
0.15
0.10
0.05
0.00
CT
NC
Prosaposin Normalised LFQ values
*
0.015
0.010
0.005
0.000
CT
NC
Hemoglobin subunit beta-2 Normalised LFQ values
ns
0.25
0.20
0.15
0.10
0.05
0.00
CT
NC
Haptoglobin Normalised LFQ values
ns
0.8
0.6
0.4
0.2
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CT
NC
Mannan-binding lectin serine protease 2 Normalised LFQ values
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0.005
0.000
CT
NC

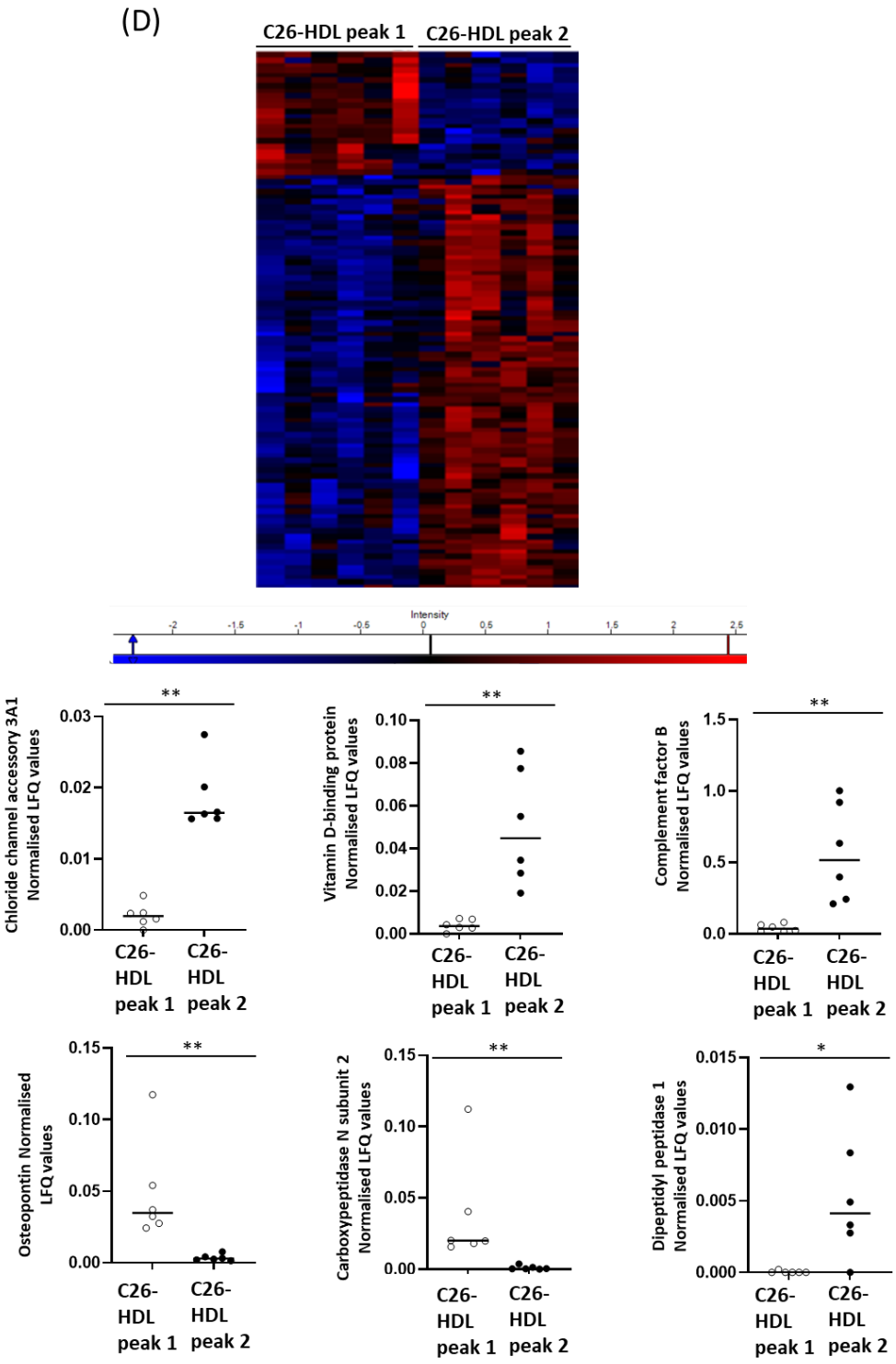


Figure 8: Heatmaps and dot plots representing proteins that are significantly different between the livers of (A) CT and C26-HDL peak 1 and (B) NC and C26-HDL peak 1 (C) CT vs NC and (D) C26-HDL peak 1 and C26-HDL peak 2**.** Dot plots show the LFQ values of the top 6 most significant proteins as determined by t-test in Perseus. Significance of LFQ values was determined in GraphPad for display purposes. p**** ≤0.0001,p***≤0.001, p** ≤0.01,p*≤0.05.

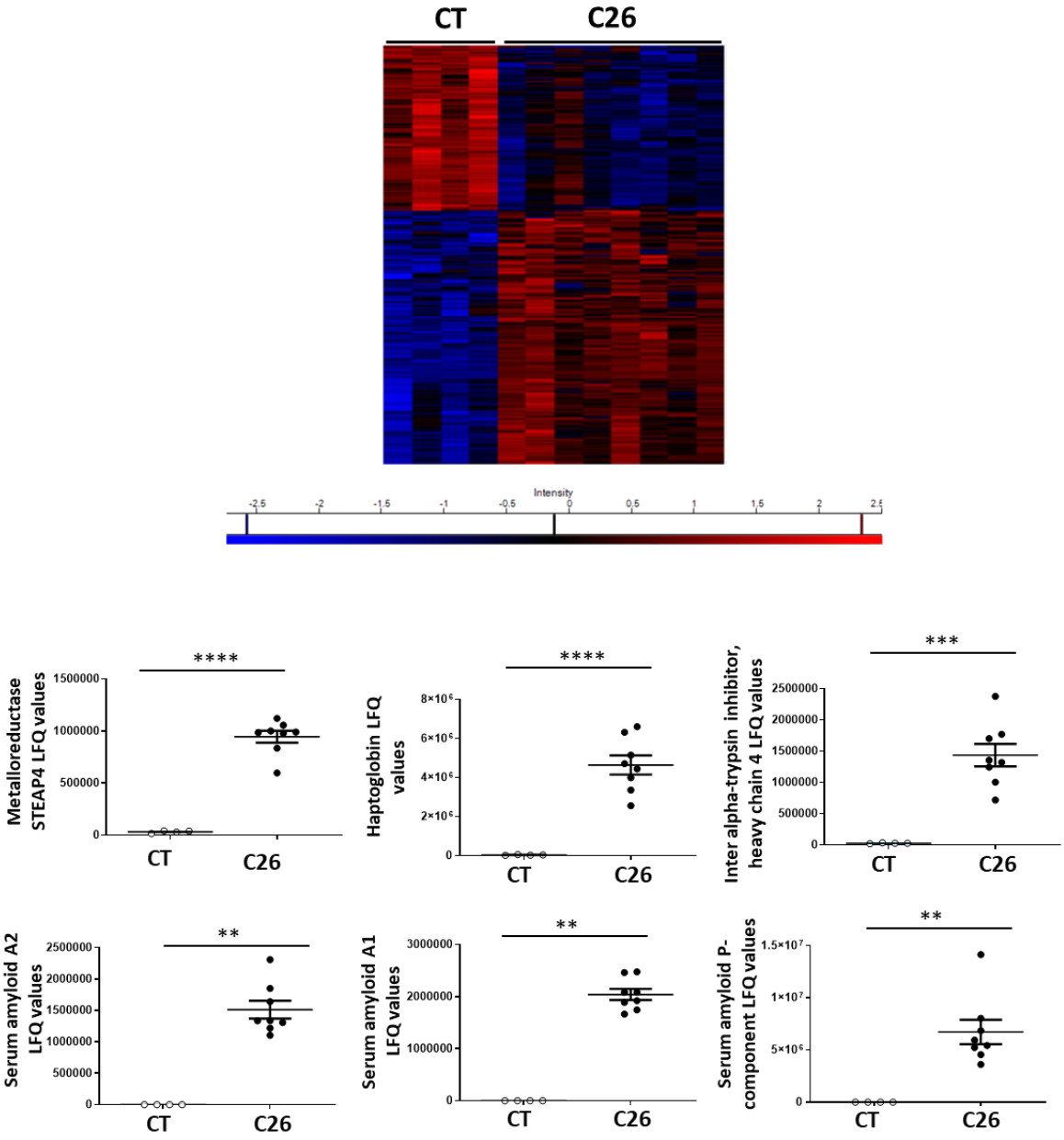
The LDL proteome is not as abundant or diverse as the HDL proteome:

Compared to the *n*=236 proteins identified on HDL, only *n*=49 proteins were identified in the pooled LDL fractions of all 3 groups, (Supplementary Table 9). C26-LDL peak 2 had the greatest number of proteins (*n*=40) followed by NC-LDL peak 2 (*n*=37) while NC-LDL peak 1 had the lowest (*n*=14). Interestingly, NC-LDL peak 1 also lacked ApoB from at least 70% of the samples, while it was present in all other groups. There were *n*=14 proteins shared among all groups including fibrinogen alpha chain (FGA), fibrinogen gamma chain (FGG), A2M, Hemoglobin subunit beta (HBB), fibronectin (FN1) and Keratin, type I cytoskeletal 9 (KRT9). C26-LDL peak 2 and NC-LDL peak 2 shared *n*=9 proteins, most of which were proteasome subunits including proteasome subunit alpha type-2 (PSMA2), proteasome subunit alpha type-4 (PSMA4), proteasome subunit alpha type (PSMA6) and proteasome subunit beta type-5 (PSMB5).

Cancer cachexia significantly affects the liver in mice:

To determine if cancer cachexia significantly affects the liver, proteomic analysis was conducted on the livers of mice with cancer cachexia (C26) (*n*=8), mice with cancer but without cachexia (NC) (*n*=7), and control mice without cancer (CT) (*n*=4). Collectively, *n*=2264 proteins were detected across the groups. When comparing the livers of CT and C26 mice (Figure 9A), n=366 proteins were significant, n=223 of which were upregulated in the C26 mice, including Serum amyloid P-component (APCS), Serum amyloid A-1 protein (SAA1), Haptoglobin (Hp), Serum amyloid A-2 protein (SAA2) and Inter alpha-trypsin inhibitor, heavy chain 4 (ITIH4), and *n*=143 of which were downregulated, including 3-ketoacyl-CoA thiolase B, peroxisomal (Acaa1b), Cytochrome P450 3A11 (Cyp3a11), Thyroid hormone-inducible hepatic protein (THRSP), and Transthyretin (TTR) (Full list available in Supplementary Table 1). When comparing the livers of NC and C26 mice (Figure 9B), *n*=690 proteins were significant, *n*=357 of which were upreglated in the C26 mice, including APCS, SAA1, SAA2, Inositol-3-phosphate synthase 1 (Isyna1), Alpha-2-macroglobulin-P (A2mp) and Serine protease inhibitor A3N (Serpina3n), and n=333 of which were downregulated including Acaa1b, TTR, THRSP and Carboxylesterase 1E (Ces1e) (Full list available in Supplementary Table 2). Interestingly, there were no significant differences between the livers of CT and NC mice. Many of the proteins identified in this study were significant in both the CT versus C26 and NC versus C26 comparison (*n*=328). Additonally, *n*=358 proteins were uniquely different in NC mice versus C26 mice and *n*=37 proteins were uniquely different in CT versus C26 mice.

(A)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C26
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1
1.5
2
2.5
Metalloreductase STEAP4 LFQ values
****
CT
C26
Haptoglobin LFQ values
****
Inter alpha-trypsin inhibitor, heavy chain 4 LFQ values
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Serum amyloid A2 LFQ values
**
Serum amyloid A1 LFQ values
**
Serum amyloid P-component LFQ values
**

(B)

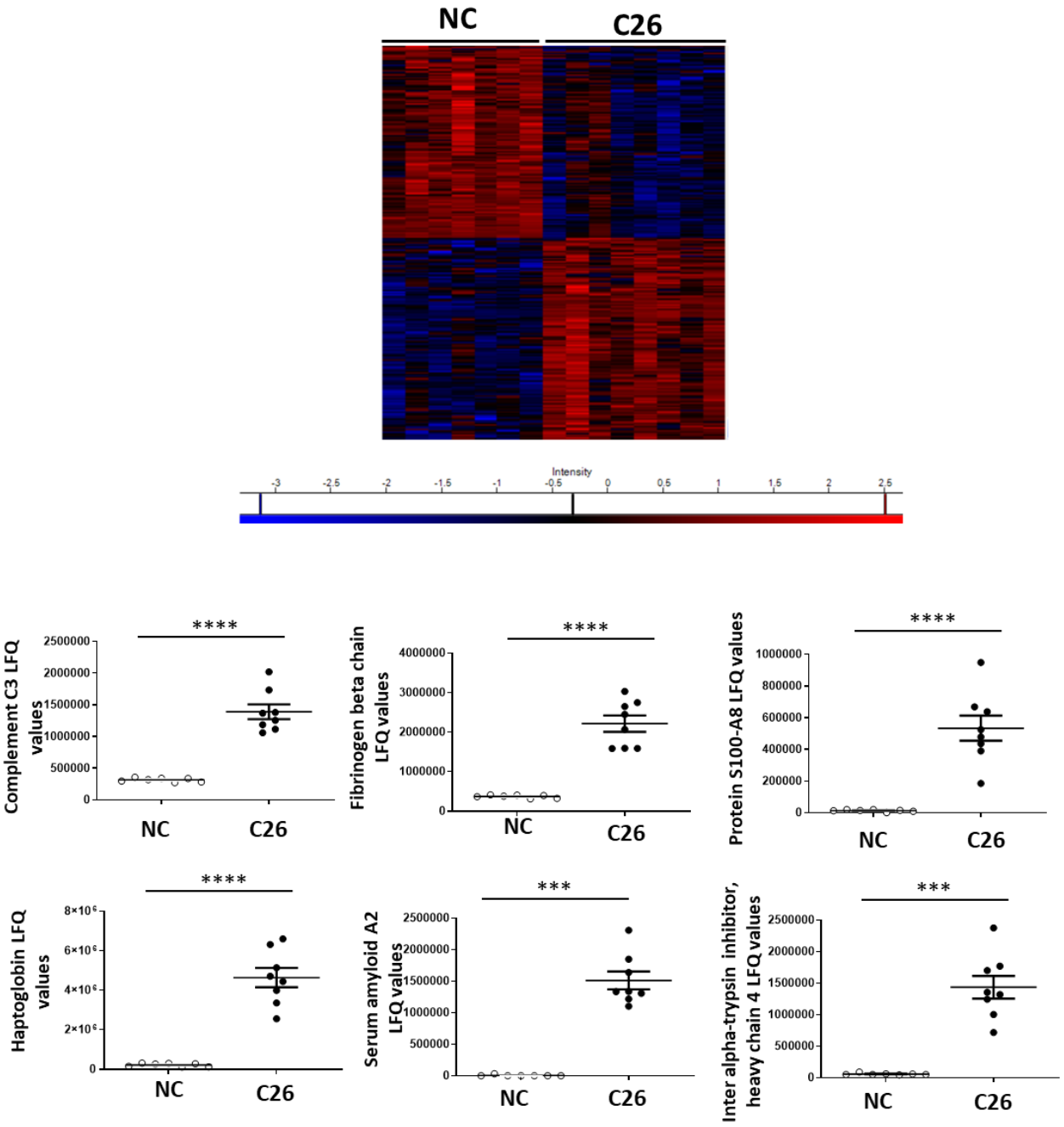


Figure 9: Heatmaps and dot plots representing proteins that are significantly different between the livers of (A) CT and C26 and (B) NC and C26. Dot plots show the LFQ values of the top 6 most significant proteins as determined by t-test in Perseus. Significance of LFQ values was determined in GraphPad for display purposes. p**** ≤0.0001,p***≤0.001,p** ≤0.01,p*≤0.05.

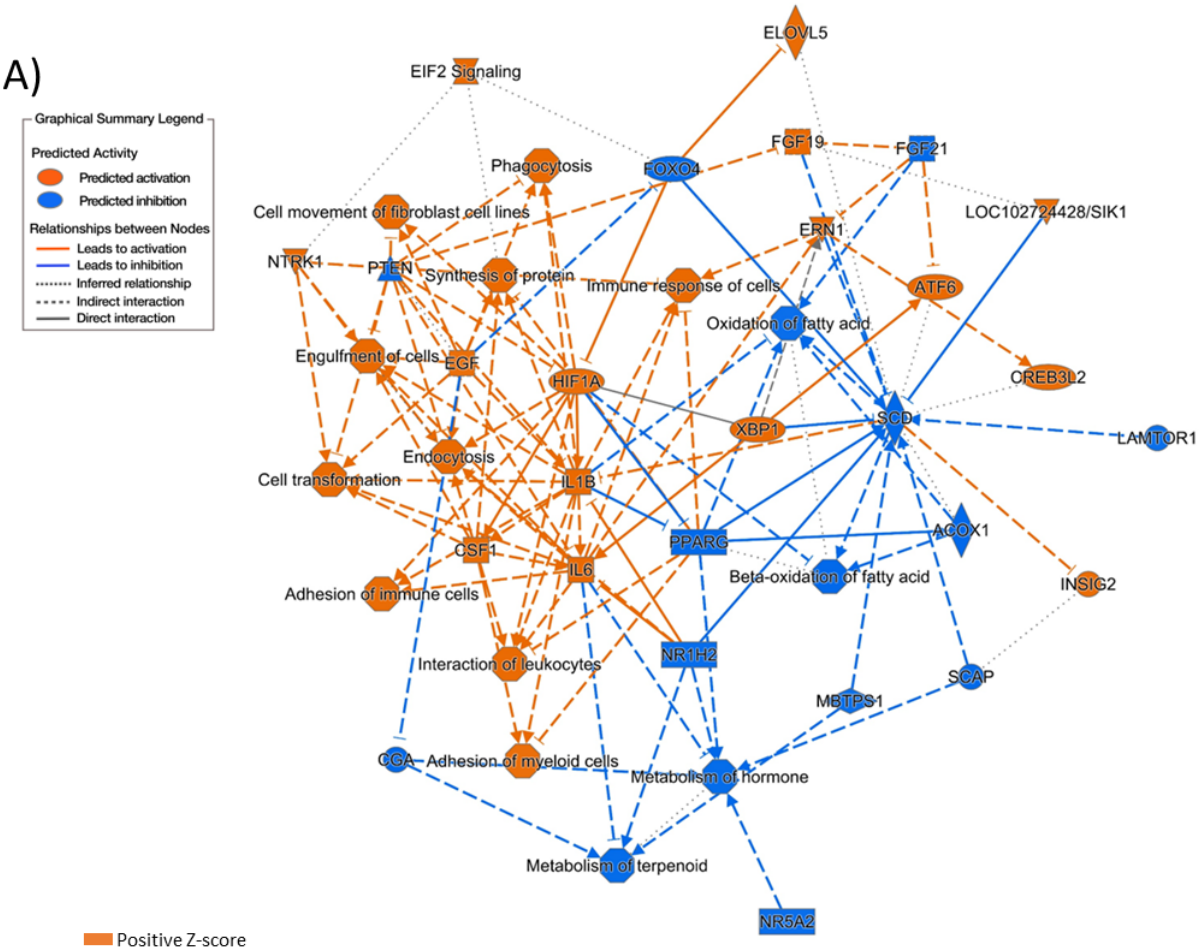
Cancer cachexia affects hepatic pathways related to protein synthesis, xenobiotic metabolism and the immune system:

To determine the pathways most affected by cachexia in the liver, all hepatic proteins identified as significant ($p<0.05$) were analysed by Ingenuity Pathway Analysis (IPA). When comparing CT vs C26 mice, *n*=934 pathways were identified by IPA (Top 100 pathways and associated proteins in Supplementary Table 3). These pathways fall into the themes of protein synthesis, immune regulation, beta-oxidation of fatty acids and hormone metabolism to name a few. Key regulators of these pathways identified by IPA include, interleukin-6 (IL-6), hypoxia-inducible factor 1-alpha (HIF1α), interleukin-1β (IL-1β), forkhead box protein O4 (FOXO4) and fibroblast growth factor 21 (FGF21). The top 20 most significantly affected pathways and graphical summary are shown in Figure 10A. SRP-dependent cotranslational protein targeting to membrane was the most significantly affected pathway (-LOG(p-value)=47.3, z-score=7.07), followed by eukaryotic translation initiation (-LOG(p-value)=41.9, z-score=6.93), eukaryotic translation elongation (-LOG(p-value)=40.9, z-score=6.56), EIF2 signaling (-LOG(p-value)=35.4, z-score=4.75) and Response of EIF2AK4 (GCN2) to amino acid deficiency (-LOG(p-value)=35.1, z-score=6.32), all of which were upregulated in cachexia. Also affected were pathways associated with xenobiotic metabolism, xenobiotic metabolism PXR signaling pathway (-LOG(p-value)=14.1, z-score=-4.38), xenobiotic metabolism signaling (-LOG(p-value)=12.7, z-score=NA) and xenobiotic metabolism CAR signaling pathway (-LOG(p-value)=12.5, z-score=-3.78) which were downregulated, with the exception of xenobiotic metabolism signaling which was undetermined. Also identified were pathways involved in the acute phase response, LPS/IL-1 mediated inhibition of RXR function (-LOG(p-value)=16.1, z-score=1.13) and acute phase response signalling (-LOG(p-value)=12.9, z-score=3.3), which were upregulated.

When comparing NC vs C26 mice, n=977 pathways were identified by IPA (Top 100 pathways and associated proteins in Supplementary Table 4), *n*=899 of which were shared with the CT vs C26 comparison. These pathways fall into the themes of cancer cell death, metastasis and virus replication, to name a few. Key regulators of these pathways identified by IPA include, IL-6, IL-1β, transforming growth factor-β (TGF-β), and Signal transducer and activator of transcription 3 (STAT3). The top 20 most significantly affected pathways and graphical summary are shown in Figure 10B. SRP-dependent cotranslational protein targeting to membrane was the most significantly affected pathway (-LOG(p-value)=47.6, z-score=7.21), followed by eukaryotic translation initiation (-LOG(p-value)=44.8, z-score=7.21), eukaryotic translation elongation (-LOG(p-value)=44.6, z-score=6.86), eukaryotic translation termination (-LOG(p-value)=38.5, z-score=6.56) and nonsense-mediated decay (-LOG(p-value)=38.1, z-score=6.78), all of which were upregulated in C26 mice. Xenobiotic metabolism pathways were also significantly downregulated in C26 mice compared to NC mice including xenobiotic

metabolism PXR signaling pathway (-LOG(p-value)=20.5, z-score=-5.19), xenobiotic metabolism signaling (-LOG(p-value)=18.5, z-score=NA), xenobiotic metabolism CAR signaling pathway (-LOG(p-value)=19.7, z-score=-4.77) and xenobiotic metabolism AHR signaling pathway (-LOG(p-value)=17.3, z-score=-4.6).

(A)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Predicted inhibition
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Leads to inhibition
Inferred relationship
Indirect interaction
Direct interaction
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EIF2 Signaling
FGF19
FGF21
Phagocytosis
FOXO4
Cell movement of fibroblast cell lines
LOC102724428/SIK1
ERN1
NTRK1
PTEN
Synthesis of protein
Immune response of cells
ATF6
Oxidation of fatty acid
Engulfment of cells
EGF
HIF1A
OREB3L2
XBP1
SCD
LAMTOR1
Endocytosis
Cell transformation
IL1B
PPARG
ACOX1
CSF1
IL6
Beta-oxidation of fatty acid
INSIG2
Adhesion of immune cells
NR1H2
Interaction of leukocytes
SCAP
MBTPS1
CGA
Adhesion of myeloid cells
Metabolism of hormone
Metabolism of terpenoid
NR5A2
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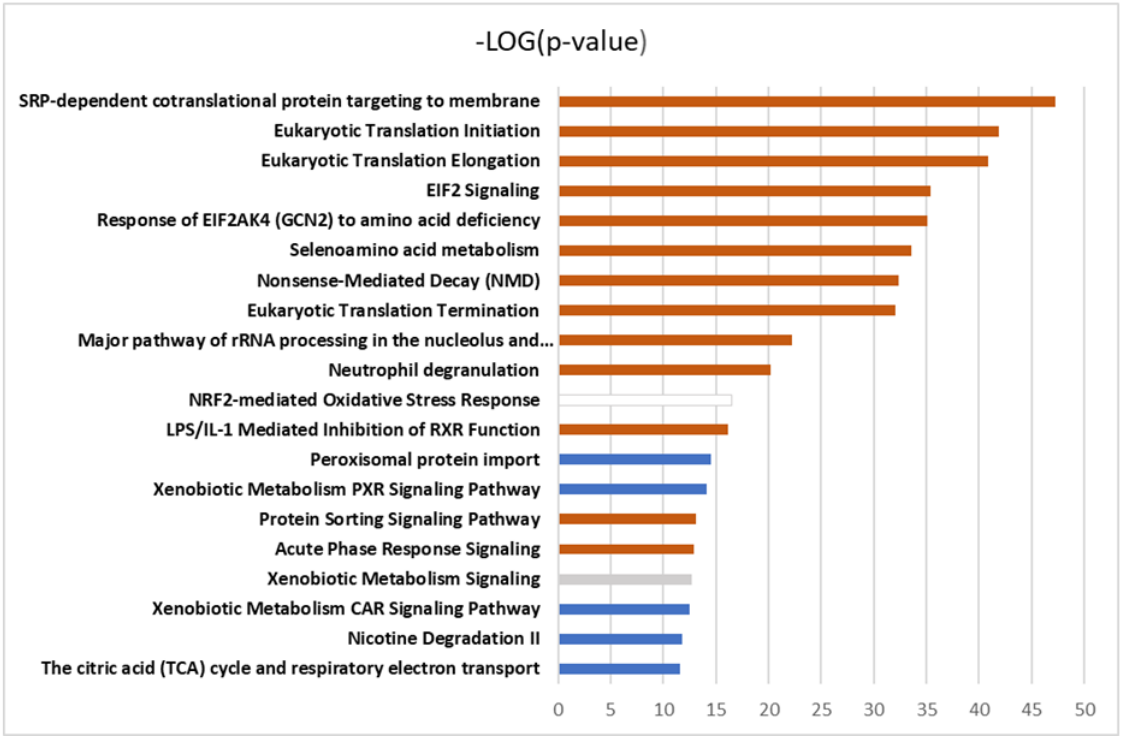
Negative Z-score
Z-score not determined


-LOG(p-value)
SRP-dependent cotranslational protein targeting to membrane
Eukaryotic Translation Initiation
Eukaryotic Translation Elongation
EIF2 Signaling
Response of EIF2AK4 (GCN2) to amino acid deficiency
Selenoamino acid metabolism
Nonsense-Mediated Decay (NMD)
Eukaryotic Translation Termination
Major pathway of rRNA processing in the nucleolus and...
Neutrophil degranulation
NRF2-mediated Oxidative Stress Response
LPS/IL-1 Mediated Inhibition of RXR Function
Peroxisomal protein import
Xenobiotic Metabolism PXR Signaling Pathway
Protein Sorting Signaling Pathway
Acute Phase Response Signaling
Xenobiotic Metabolism Signaling
Xenobiotic Metabolism CAR Signaling Pathway
Nicotine Degradation II
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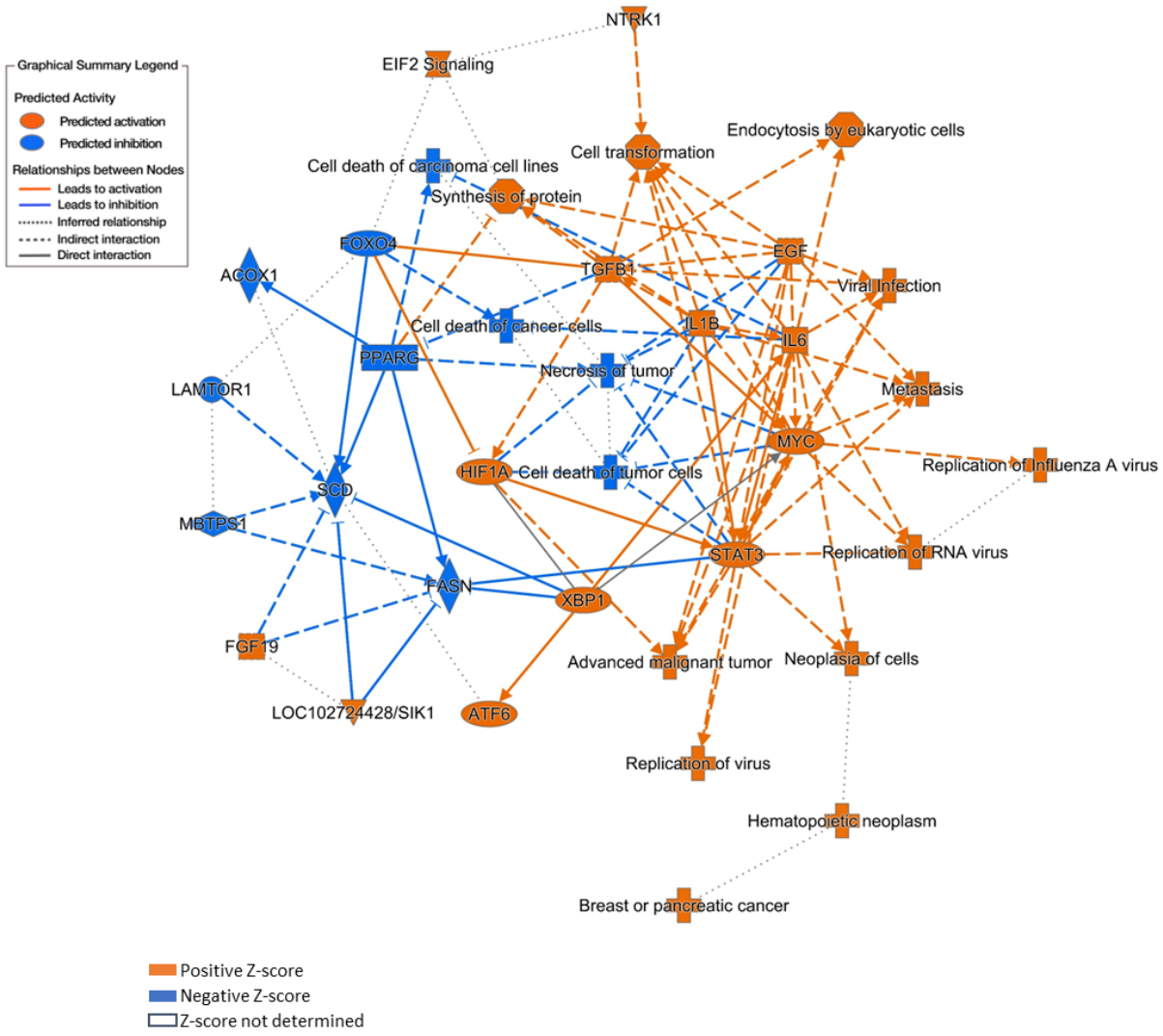
15
20
25
30
35
40
45
50

(B)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Predicted inhibition
Relationships between Nodes
Leads to activation
Leads to inhibition
Inferred relationship
Indirect interaction
Direct interaction
NTRK1
EIF2 Signaling
Endocytosis by eukaryotic cells
Cell transformation
Cell death of carcinoma cell lines
Synthesis of protein
FOXO4
EGF
TGFB1
ACOX1
Viral Infection
Cell death of cancer cells
IL1B
IL6
PPARG
Necrosis of tumor
LAMTOR1
Metastasis
MYC
Replication of Influenza A virus
HIF1A
Cell death of tumor cells
SCD
MBTPS1
STAT3
Replication of RNA virus
FASN
XBP1
FGF19
Advanced malignant tumor
Neoplasia of cells
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ATF6
Replication of virus
Hematopoietic neoplasm
Breast or pancreatic cancer
Positive Z-score
Negative Z-score
Z-score not determined

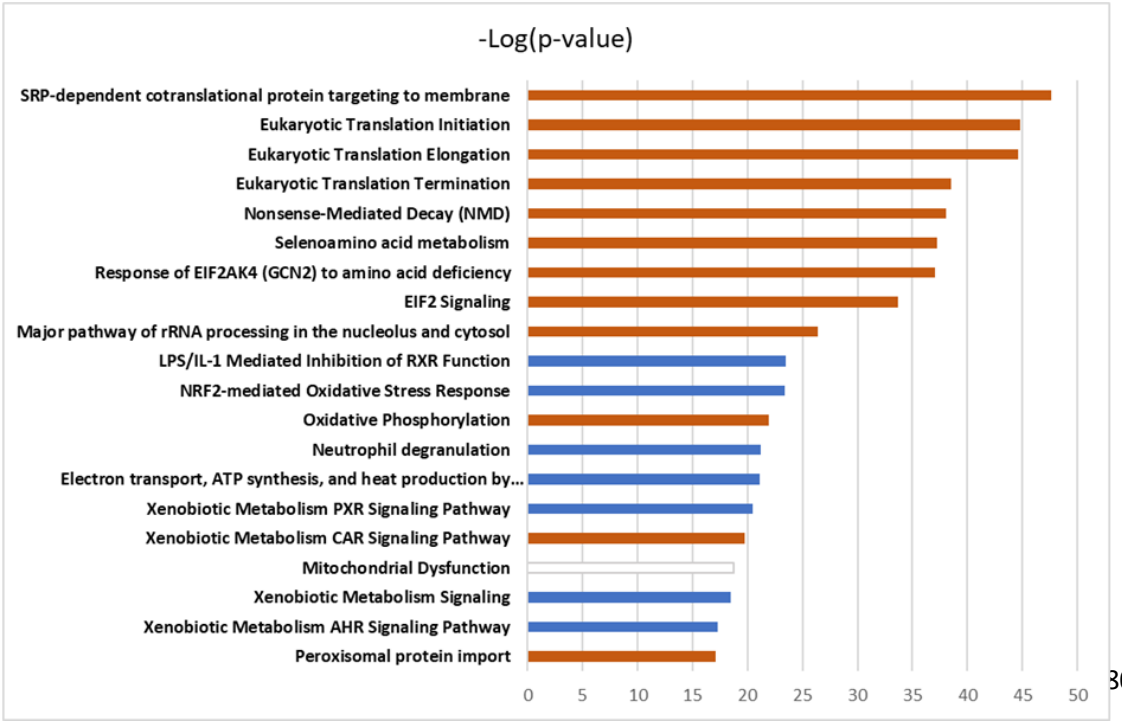
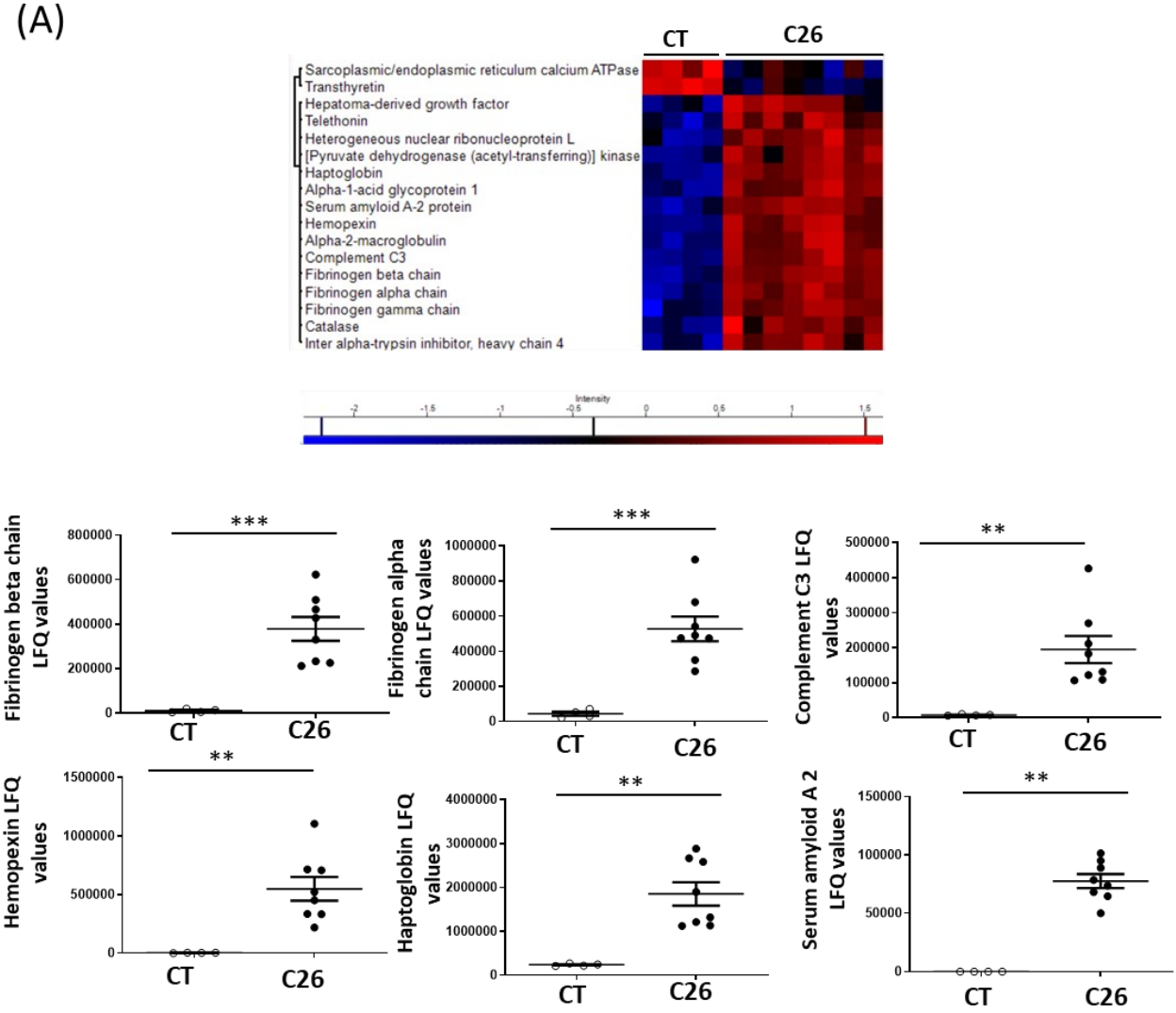

-Log(p-value)
SRP-dependent cotranslational protein targeting to membrane
Eukaryotic Translation Initiation
Eukaryotic Translation Elongation
Eukaryotic Translation Termination
Nonsense-Mediated Decay (NMD)
Selenoamino acid metabolism
Response of EIF2AK4 (GCN2) to amino acid deficiency
EIF2 Signaling
Major pathway of rRNA processing in the nucleolus and cytosol
LPS/IL-1 Mediated Inhibition of RXR Function
NRF2-mediated Oxidative Stress Response
Oxidative Phosphorylation
Neutrophil degranulation
Electron transport, ATP synthesis, and heat production by...
Xenobiotic Metabolism PXR Signaling Pathway
Xenobiotic Metabolism CAR Signaling Pathway
Mitochondrial Dysfunction
Xenobiotic Metabolism Signaling
Xenobiotic Metabolism AHR Signaling Pathway
Peroxisomal protein import
0
5
10
15
20
25
30
35
40
45
50

Figure 10: Graphical summary of affected pathways and the top 20 most significantly affected pathways when comparing the livers of (A) CT versus C26 mice and (B) NC versus C26 mice. Created using QIAGENs Ingenuity Pathway Analysis (IPA). Significant pathways were determined using IPA analysis on the significant proteins as determined by t-test ($p<0.05$) in Perseus. Orange indicates pathways that are upregulated (positive z-score), blue indicates pathways that are downregulated (negative z-score) and white indicates pathways that could not be determined as up or downregulated (z-score not generated).

Cancer cachexia significantly affects the muscle in mice:

To determine the effects of cancer cachexia on the proteome of gastrocnemius muscle, proteomic analysis was conducted on the gastrocnemius of C26, NC and CT mice. Collectively, *n*=826 proteins were detected across the 3 groups. When comparing CT and C26 mice (Figure 11A), *n*=17 proteins were significant, *n*=2 of which were downregulated in C26 mice, sarcoplasmic/endoplasmic reticulum calcium ATPase 1 (Atp2a1) and TTR, and n=15 of which were upregulated including hepatoma-derived growth factor (HDGF), telethonin (TCAP), HP, Alpha-1-acid glycoprotein 1 (ORM1), SAA2 and fibrinogen beta chain (FGB). When comparing NC and C26 mice (Figure 11B), n=19 proteins were significant, *n*=3 of which were downregulated in C26 mice, ras-related protein Rab-10 (RAB10), TTR and major urinary protein 1 (MUP1) and *n*=16 proteins were upregulated including TCAP, A2M, serotransferrin (TF), FGB and serine protease inhibitor A3N (SERPINA3N). There were no differences between the gastrocenimus muscle of CT and NC mice. In total, *n*=14 proteins were significant in the CT and C26 comparison and the NC and C26 comparison. The CT and C26 comparison identified *n*=3 unique proteins HDGF, heterogeneous nuclear ribonucleoprotein L (HNRNPL) and Atp2a1 and the NC and C26 comparison identified *n*=5 unique proteins, major urinary protein 15 (MUP15), RAB10, 14-3-3 protein zeta/delta (Ywhaz), SERPINA3N and TF.

(A)


CT
C26
Sarcoplasmic/endoplasmic reticulum calcium ATPase
Transthyretin
Hepatoma-derived growth factor
Telethonin
Heterogeneous nuclear ribonucleoprotein L
[Pyruvate dehydrogenase (acetyl-transferring)] kinase
Haptoglobin
Alpha-1-acid glycoprotein 1
Serum amyloid A-2 protein
Hemopexin
Alpha-2-macroglobulin
Complement C3
Fibrinogen beta chain
Fibrinogen alpha chain
Fibrinogen gamma chain
Catalase
Inter alpha-trypsin inhibitor, heavy chain 4
Intensity
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-0.5
0
0.5
1
1.5
Fibrinogen beta chain LFQ values
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600000
400000
200000
0
***
CT
C26
Fibrinogen alpha chain LFQ values
1000000
800000
600000
400000
200000
0
***
CT
C26
Complement C3 LFQ values
500000
400000
300000
200000
100000
0
**
CT
C26
Hemopexin LFQ values
1500000
1000000
500000
0
**
CT
C26
Haptoglobin LFQ values
4000000
3000000
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**
CT
C26
Serum amyloid A 2 LFQ values
150000
100000
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**
CT
C26

(B)

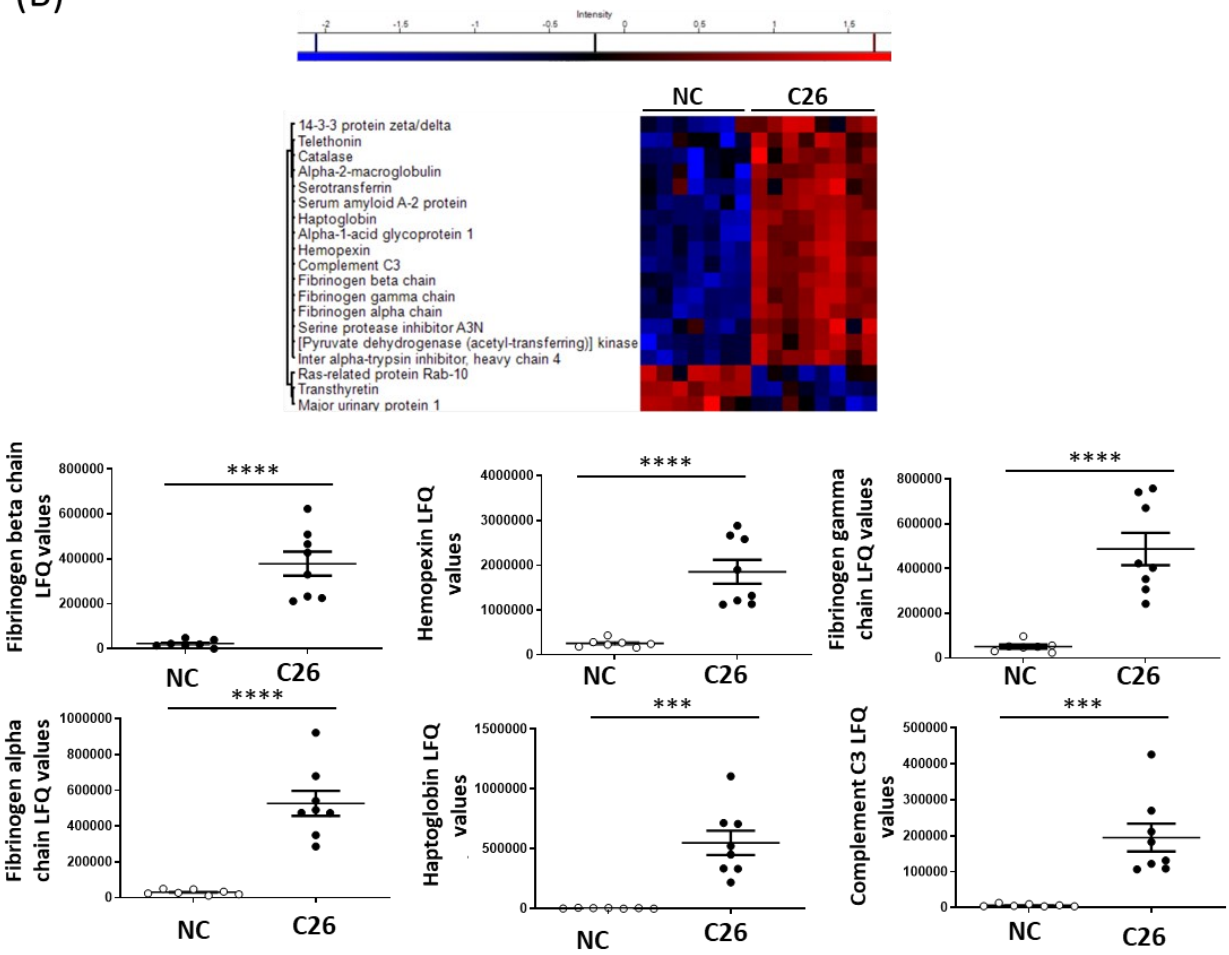


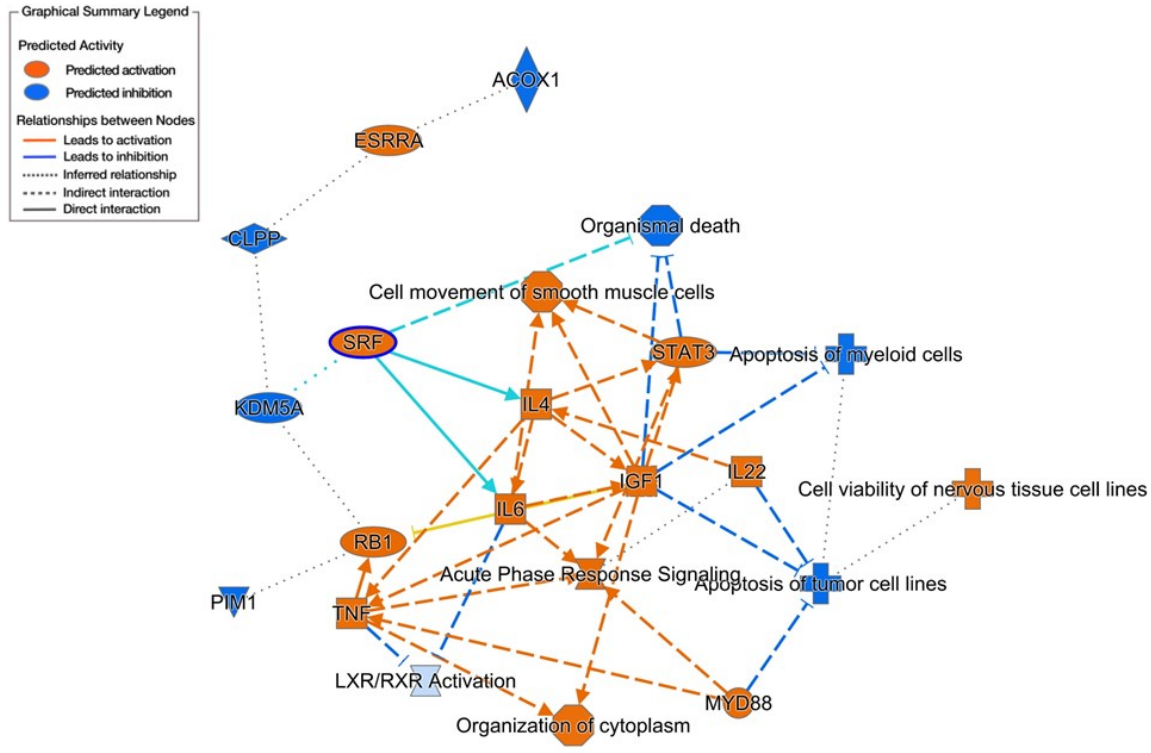
Figure 11: Heatmaps and dot plots representing the proteins that are significantly different between the livers of (A) CT and C26 and (B) NC and C6. Dot plots show the LFQ values of the top 6 most significant proteins as determined by t-test in Perseus. Significance of LFQ values was determined in GraphPad for display purposes. p**** ≤0.0001, p***≤0.001, p** ≤0.01, p*≤0.05.

Cancer cachexia affects pathways related to protein synthesis, acute phase response and apoptosis in the gastrocnemius:

To determine the pathways most affected by cachexia in the gastrocnemius, all proteins identified as significant ($p<0.05$) were analysed by IPA. When comparing CT vs C26 mice, *n*=394 pathways were identified by IPA (Top 100 pathways and associated proteins in Supplementary Table 5). These pathways fall into the themes of the acute phase response, apoptosis and protein synthesis. Key regulators of these pathways identified by IPA include, IL-6, STAT3, interleukin-4 (IL-4) and insulin growth factor 1 (IGF1). The top 20 most significantly affected pathways and graphical summary are shown in Figure 12A. acute phase response signalling was the most significantly affected pathway (-LOG(p-value)=15.8, z-score=2.53), followed by LXR/RXR activation (-LOG(p-value)=13.5, z-score=-0.3), DHCR24 signaling pathway (-LOG(p-value)=10.8, z-score=-0.30), striated muscle contraction (-LOG(p-value)=10.1, z-score=1.13) and response to elevated platelet cytosolic Ca2+ (-LOG(p-value)=7.39, z-score=1.14). Also identified were pathways involved in protein synthesis such as eukaryotic translation elongation (-LOG(p-value)=4.46, z-score=-2.24), eukaryotic translation termination (-LOG(p-value)=4.58, z-score=-2.24) and post-translational protein phosphorylation (-LOG(p-value)=6.94, z-score=-0.38) and pathways involved in coagulation including coagulation system (-LOG(p-value)=5.17, z-score=2), extrinsic prothrombin activation pathway (-LOG(p-value)=4.48, z-score= undetermined) and intrinsic prothrombin activation pathway. Additionally, the cachexia signalling pathway (-LOG(p-value)=6.04, z-score=2.53), and senescence pathway were identified (-LOG(p-value)=2.9, z-score=2.12).

When comparing NC vs C26 mice, *n*=294 pathways were identified by IPA (Top 100 pathways and associated proteins in Supplementary Table 4), *n*=239 of which were shared with the CT vs C26 comparison (Top 100 pathways and associated proteins in Supplementary Table 6). These pathways fall into themes such as acute phase response signalling, protein synthesis and neoplasia of cells. Key regulators of these pathways identified by IPA include IL-6, interleukin-22 (IL-22), PPARGC1A and interferon gamma (INFG). The top 20 most significantly affected pathways and graphical summary are shown in Figure 12B. Acute phase response signalling (-LOG(p-value)=15.7, z-score=2.83) was the most significant pathway followed by LXR/RXR activation (-LOG(p-value)=11.4, z-score=1.26), response to elevated platelet cytosolic Ca2+ (-LOG(p-value)=11, z-score=3.16), DHCR24 signaling pathway (-LOG(p-value)=10.3, z-score=1.26) and intrinsic prothrombin activation pathway (-LOG(p-value)=8.6, z-score=0.45). Other pathways include glycogen metabolism (-LOG(p-value)=5.37, z-score=-1) and neutrophil degranulation (-LOG(p-value)=5.06, z-score=-0.33). While not in the top 20 most signifcantly affected pathways, the cachexia signalling pathway was also identified (-LOG(p-value)=4.88, z-score=1.14).

(A)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Predicted inhibition
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Leads to inhibition
Inferred relationship
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Direct interaction
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ESRRA
CLPP
Organismal death
Cell movement of smooth muscle cells
SRF
STAT3
Apoptosis of myeloid cells
KDM5A
IL4
IGF1
IL22
Cell viability of nervous tissue cell lines
IL6
RB1
Acute Phase Response Signaling
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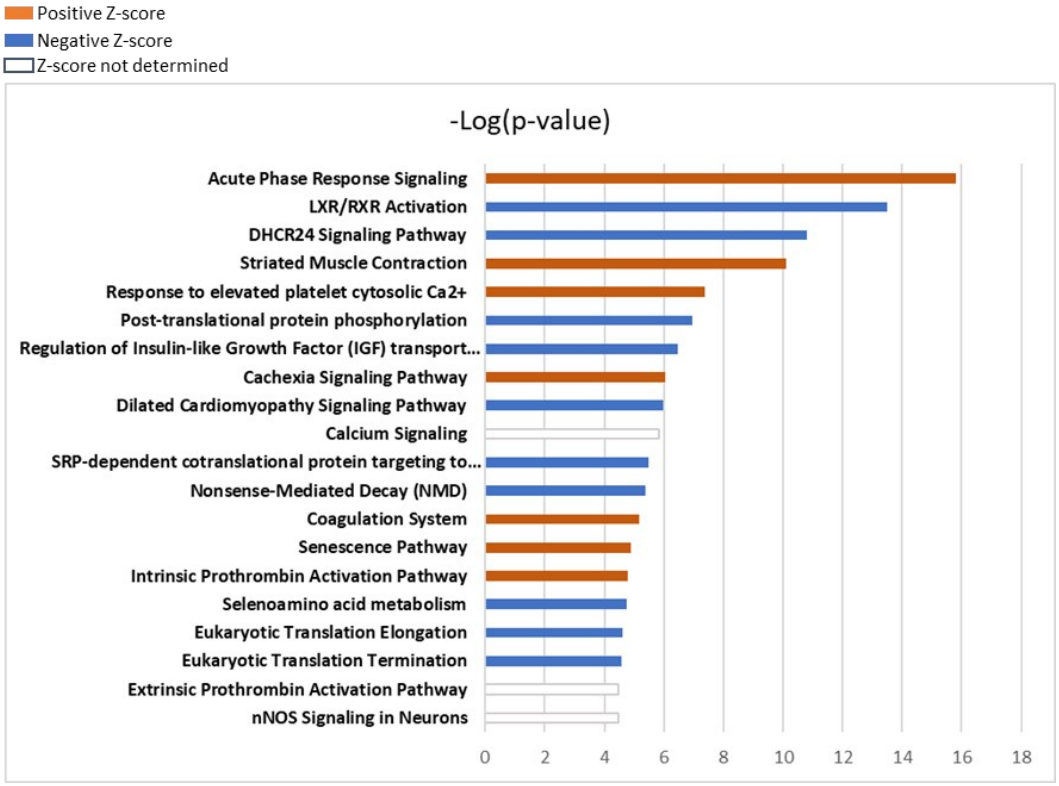
TNF
LXR/RXR Activation
MYD88
Organization of cytoplasm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DHCR24 Signaling Pathway
Striated Muscle Contraction
Response to elevated platelet cytosolic Ca2+
Post-translational protein phosphorylation
Regulation of Insulin-like Growth Factor (IGF) transport...
Cachexia Signaling Pathway
Dilated Cardiomyopathy Signaling Pathway
Calcium Signaling
SRP-dependent cotranslational protein targeting to...
Nonsense-Mediated Decay (NMD)
Coagulation System
Senescence Pathway
Intrinsic Prothrombin Activation Pathway
Selenoamino acid metabolism
Eukaryotic Translation Elongation
Eukaryotic Translation Termination
Extrinsic Prothrombin Activation Pathway
nNOS Signaling in Neurons
0
2
4
6
8
10
12
14
16
18

(B)

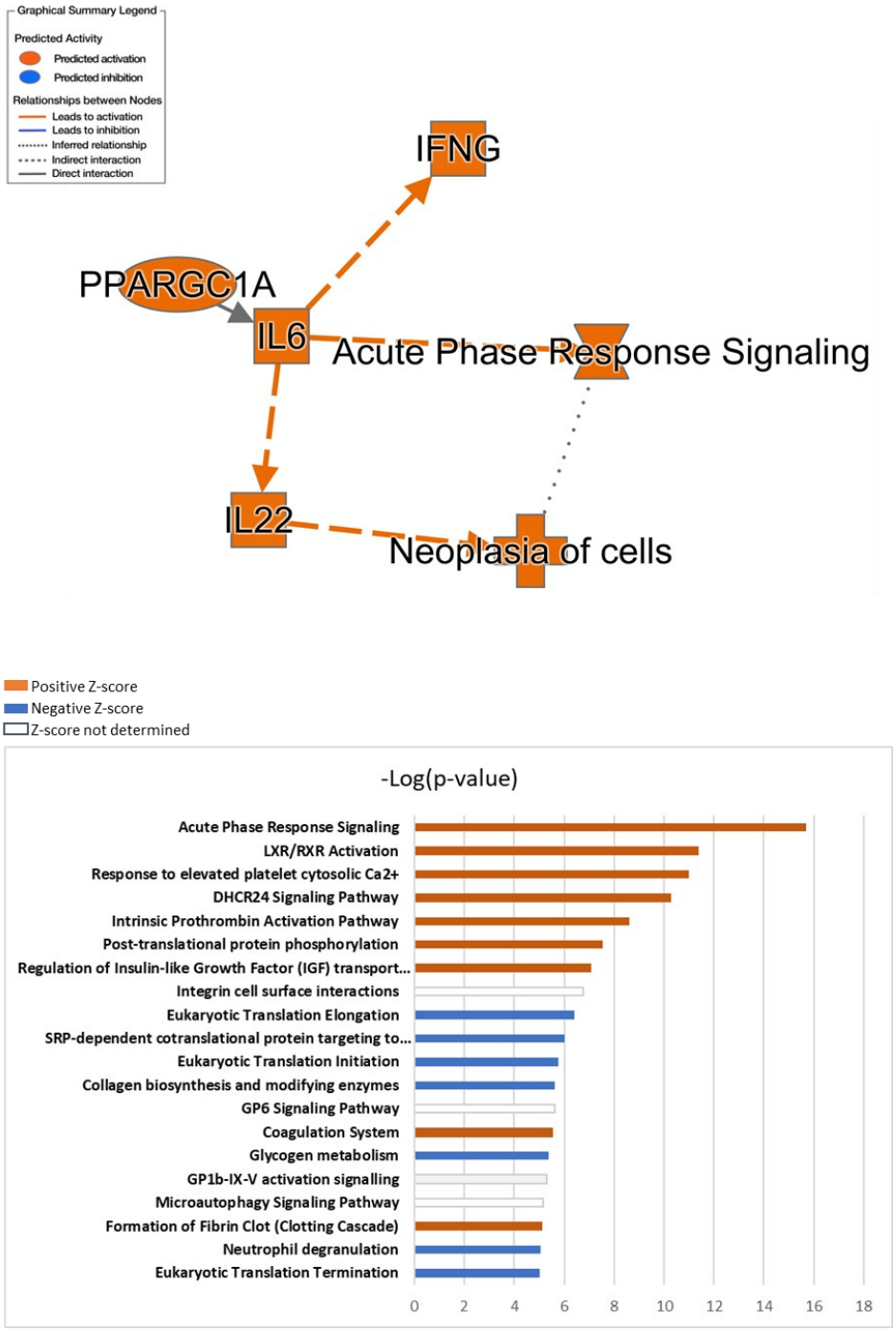


Figure 12: Graphical summary of affected pathways and the top 20 most significantly affected pathways when comparing the gastrocnemius of (A) CT versus C26 mice and (B) NC versus C26 mice**.** Created using QIAGENs Ingenuity Pathway Analysis (IPA). Significant pathways were determined using IPA analysis on the significant proteins as determined by t-test ($p<0.05$) in Perseus. Orange indicates pathways that are upregulated (positive z-score), blue indicates pathways that are downregulated (negative z-score) and white indicates pathways that could not be determined as up or downregulated (z-score not generated).

Proteins in the HDL proteome correlate with changes in body weight, liver, muscle and adipose tissue weights (%iBW):

To investigate the relationship between body weight change (Δ bodyweight), liver, muscle and adipose tissue, the final weights of tissue in grams (g) was expressed as a percentage of the initial body weight (%iBW) and correlated with proteins from the HDL proteome (

Table 6). Δ bodyweight correlated with *n*=65 proteins, *n*=38 of which negatively correlated including immunoglobulin heavy constant gamma 1 (IGHG1) (r= -0.8407, p=0.0006), inter-alpha-trypsin inhibitor heavy chain H3 (ITIH3) (r= -0.8107, p=0.0004), serum amyloid A-2 (SAA2) (r= -0.8059, p=0.0003) and clusterin (CLU) (r= -0.7941, p=0.0004), and *n*=27 of which were positively correlated including complement component 7 (C7) (r= 0.8176, p=0.0002), phosphatidylinositol-glycan-specific phospholipase D (GPLD1) (r= 0.818, p=0.0001), prosaposin (PSAP) (r= 0.8216, p=0.0019) and Cadherin-13 (CDH13) (r= 0.8481, p=0.0078).

The liver (%iBW) correlated with *n*=18 proteins, *n*=2 of which were negatively correlated, extracellular superoxide dismutase (SOD3) (r= -0.7601, p=0.0107), stress-induced-phosphoprotein 1 (STIP1) (r= -0.5934, p=0.036) and *n*=16 of which were positively correlated including immunoglobulin heavy variable 6-3 (IGHV6-3) (r= 0.8538, p=0.0305), vasorin (VASN) (r= 0.8812, p=0.0483), ig kappa chain V-V region HP 93G7 (P01645) (r= 0.8929, p=0.0123), Ig kappa chain V-III region PC 2413 (IGKV7-33) (r= 0.9237, p=0.025).

The gastrocnemius (%iBW) correlated with *n*=77 proteins, of these *n*=52 were negatively correlated including 78 kDa glucose-regulated protein (HSPA5) (r= -0.8418, p=0.0003), N-acetylmuramoyl-L-alanine amidase (PGLYRP2) (r= -0.811, p=0.0007), afamin (AFM) (r= -0.7765, p=0.0007) and serum amyloid P-component (APCS) (r= -0.7765, p=0.0007) and *n*=25 were positively correlated including murinoglobulin-1 (MUG1) (r= 0.7971, p=0.0004), carboxypeptidase N subunit 2 (CPN2) (r= 0.8141, p=0.0007), C7 (r= 0.8206, p=0.0002), mannose-binding protein A (MBL1) (r= 0.8213, p=0.0124)

The tibialis (%iBW) correlated with *n*=70 proteins, *n*=47 of which were negatively correlated including HSPA5 (r= -0.7978, p=0.001), AFM (r= -0.7853, p=0.0005), CLU (r= -0.7706, p=0.0008) and vitronectin VTN (r= -0.7676, p=0.0008) and n=23 of which were positively correlated including C7 (r= 0.7853, p=0.0005), leukaemia inhibitory factor receptor (LIFR) (r= 0.7857, p=0.0005), MUG1 (r= 0.7912, p=0.0004) and Plasma kallikrein (KLKB1) (r= 0.8147, p=0.0002).

Brown adipose tissue (BAT) (%iBW) correlated with n=62 proteins, n=35 of these were negatively correlated including granulins (GRN) (r= -0.8344, p=0.0007), ITIH3 (r= -0.7964, p=0.0006), Apolipoprotein C-III (APOC3) (r= -0.7571, p=0.0016) and serum amyloid A-1 (SAA1) (r= -0.7471, p=0.0013) and n=27 of these were positively correlated including adenylosuccinate lyase (ADSL) (r= 0.7939, p=0.0088), inter-alpha-trypsin inhibitor heavy chain H2 (ITIH2) (r=0.8088, p=0.0003), Hepatocyte growth factor activator (HGFAC) (r= 0.8206, p=0.0002) and H-2 class I histocompatibility antigen (H2-Q10) (r= 0.833, p=0.0004).

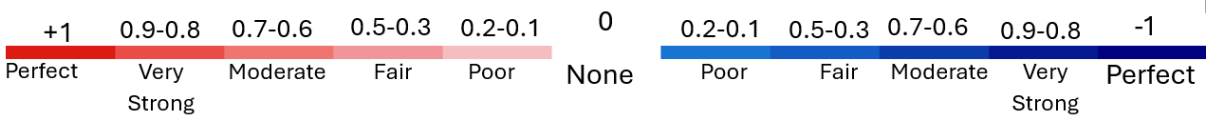
Subcutaneous adipose tissue (SAT) (%iBW) correlated with n=54 proteins, n=31 of which were negatively correlated including APOC3 (r= -0.7786, p=0.001), ITIH3 (r= -0.7393, p=0.0023), serum amyloid A-4 (SAA4) (r= -0.7265, p=0.002) and CLU (r= -0.7235, p=0.0021) and n=23 of which were positively correlated HGFAC (r= 0.7912, p=0.0004), KLKB1 (r= 0.7971, p=0.0004), H2-Q10 (r= 0.8198, p=0.0006) and LIFR (r= 0.8329, p=0.0001).

Table 6: Correlation analysis of HDL proteins and the Δ Bodyweight (%iBW), and weight of the liver, gastrocnemius, tibialis, BAT and SAT (%iBW)

	Δ Bodyweight (%iBW)		Liver (%iBW)		Gastrocnemius (%iBW)		Tibialis (%iBW)		BAT (%iBW)		SAT (%iBW)	
	r-value	p-value	r-value	p-value	r-value	p-value	r-value	p-value	r-value	p-value	r-value	p-value
2210010C04Rik	-0.355	0.286	0.418	0.203	-0.682	0.025	-0.546	0.088	-0.391	0.237	-0.346	0.299
IGHV8-12	-0.521	0.049	0.364	0.182	-0.589	0.023	-0.543	0.039	-0.321	0.243	-0.346	0.206
IGKV6-15	-0.564	0.096	0.321	0.368	-0.709	0.027	-0.576	0.088	-0.491	0.155	-0.37	0.296
A2M	0.667	0.005	0.418	0.108	0.468	0.068	0.539	0.031	0.715	0.002	0.584	0.018
A2MP	-0.659	0.017	0.451	0.125	-0.769	0.003	-0.692	0.011	-0.643	0.021	-0.599	0.034
ACTB	-0.577	0.081	0.569	0.086	-0.47	0.17	-0.453	0.188	-0.662	0.037	-0.517	0.126
ADSL	0.806	0.007	-0.079	0.838	0.552	0.105	0.479	0.166	0.794	0.009	0.709	0.027
AFM	-0.688	0.004	0.121	0.656	-0.777	0.001	-0.785	0.001	-0.562	0.026	-0.506	0.048
AHSG	-0.35	0.201	0.029	0.923	-0.468	0.081	-0.554	0.035	-0.393	0.149	-0.389	0.153
ALDOA	-0.637	0.022	0.247	0.415	-0.775	0.003	-0.615	0.028	-0.55	0.055	-0.571	0.045
AMBP	-0.682	0.005	-0.141	0.602	-0.459	0.076	-0.553	0.029	-0.656	0.007	-0.582	0.02
APCS	-0.762	0.001	-0.147	0.586	-0.777	0.001	-0.694	0.004	-0.674	0.005	-0.641	0.009
APOA2	0.665	0.006	0.135	0.617	0.594	0.017	0.471	0.068	0.579	0.021	0.591	0.018
APOA4	0.685	0.004	0.477	0.064	0.379	0.148	0.477	0.064	0.518	0.042	0.612	0.014
APOC3	-0.746	0.002	-0.036	0.903	-0.579	0.026	-0.536	0.042	-0.757	0.002	-0.779	0.001
APOC4	0.527	0.039	0.106	0.697	0.391	0.135	0.427	0.101	0.506	0.048	0.568	0.024
APOD	0.424	0.104	0.009	0.978	0.353	0.18	0.277	0.299	0.427	0.101	0.512	0.045
APOE	0.747	0.001	0.074	0.788	0.659	0.007	0.632	0.01	0.662	0.007	0.556	0.028
APOM	0.293	0.272	0.071	0.793	0.272	0.309	0.313	0.237	0.525	0.037	0.274	0.304
B2M	0.521	0.068	0.204	0.503	0.581	0.037	0.337	0.26	0.396	0.181	0.444	0.129
C1QC	0.064	0.86	0.791	0.006	-0.018	0.967	0.191	0.577	-0.045	0.903	0.009	0.99
C1S1	0.841	<0.0001	0.135	0.617	0.65	0.008	0.679	0.005	0.777	0.001	0.779	0.001
C2 *	0.029	0.951	0.83	0.021	0.044	0.926	0.459	0.301	-0.298	0.516	-0.116	0.805
C3	-0.671	0.004	-0.021	0.939	-0.661	0.005	-0.584	0.018	-0.519	0.04	-0.592	0.016
C4B	-0.536	0.032	0.084	0.757	-0.505	0.046	-0.49	0.054	-0.358	0.174	-0.485	0.057
C7	0.818	<0.0001	0.109	0.689	0.821	<0.0001	0.785	0.001	0.7	0.003	0.644	0.009
C8A	-0.488	0.057	0.079	0.771	-0.535	0.035	-0.518	0.042	-0.435	0.094	-0.412	0.114
C8B	-0.45	0.082	0.329	0.213	-0.597	0.017	-0.5	0.051	-0.356	0.176	-0.397	0.129
C8G	-0.555	0.053	0.253	0.404	-0.615	0.028	-0.588	0.038	-0.555	0.053	-0.571	0.045
CA2 *	0.214	0.619	0.81	0.022	0.024	0.977	-0.071	0.882	0.071	0.882	0	>0.999
CAT	-0.506	0.048	-0.015	0.961	-0.512	0.045	-0.632	0.01	-0.432	0.096	-0.356	0.176
CES1B	-0.264	0.435	0.482	0.138	-0.646	0.037	-0.655	0.034	-0.236	0.485	0.045	0.903
CES1C	-0.474	0.066	0.127	0.641	-0.603	0.015	-0.568	0.024	-0.385	0.141	-0.321	0.226
CFHR1	-0.582	0.02	0.097	0.721	-0.571	0.023	-0.559	0.027	-0.4	0.126	-0.462	0.074
CFP	0.632	0.024	0.401	0.176	0.56	0.05	0.467	0.11	0.445	0.13	0.434	0.141
CLCA3A1	-0.762	0.006	-0.098	0.766	-0.713	0.012	-0.699	0.014	-0.664	0.022	-0.699	0.014
CLU	-0.794	<0.001	-0.015	0.961	-0.762	0.001	-0.771	0.001	-0.706	0.003	-0.724	0.002
CP	-0.471	0.068	0.303	0.253	-0.685	0.004	-0.568	0.024	-0.535	0.035	-0.538	0.034
CPB2	-0.436	0.183	0.373	0.261	-0.627	0.044	-0.464	0.155	-0.455	0.164	-0.482	0.138
CPN1	0.735	0.002	0.109	0.689	0.718	0.002	0.735	0.002	0.624	0.012	0.559	0.027
CPN2	0.882	<0.0001	0.244	0.397	0.814	0.001	0.898	<0.0001	0.724	0.005	0.687	0.008
CSF1R	0.75	0.001	-0.088	0.746	0.641	0.009	0.641	0.009	0.768	0.001	0.759	0.001
EGFR	-0.65	0.008	0.068	0.805	-0.671	0.006	-0.597	0.017	-0.509	0.046	-0.538	0.034
F10	-0.224	0.404	0.441	0.089	-0.259	0.332	-0.259	0.332	-0.512	0.045	-0.397	0.129
F13B	-0.141	0.602	0.606	0.015	-0.124	0.649	-0.038	0.891	-0.203	0.45	-0.224	0.404
F2	-0.641	0.009	0.059	0.831	-0.738	0.002	-0.679	0.005	-0.594	0.017	-0.559	0.027
F9	-0.367	0.197	0.209	0.473	-0.574	0.035	-0.591	0.029	-0.481	0.084	-0.371	0.192
FBLN1	0.665	0.006	0.021	0.943	0.753	0.001	0.694	0.004	0.618	0.013	0.515	0.044
FCN1&	0.475	0.234	0.638	0.089	0.701	0.053	0.514	0.192	-0.649	0.082	-0.583	0.13
GC	-0.671	0.006	0.121	0.656	-0.732	0.002	-0.715	0.003	-0.618	0.013	-0.585	0.019
GM20547	-0.674	0.005	0.032	0.908	-0.768	0.001	-0.709	0.003	-0.527	0.039	-0.541	0.033

GPLD1	0.818	<0.0001	0.185	0.492	0.635	0.008	0.679	0.004	0.756	0.001	0.771	0.001
GRN	-0.741	0.006	0.014	0.966	-0.609	0.035	-0.573	0.052	-0.834	0.001	-0.718	0.009
GSN	-0.404	0.137	0.446	0.097	-0.536	0.042	-0.55	0.036	-0.261	0.347	-0.264	0.34
H2-Q10	0.785	0.001	0.007	0.988	0.732	0.004	0.662	0.012	0.833	<0.0001	0.82	0.001
HBA	-0.215	0.423	0.092	0.733	-0.018	0.949	-0.085	0.755	-0.505	0.046	-0.33	0.212
HGFAC	0.8	<0.0001	-0.115	0.672	0.659	0.007	0.671	0.006	0.821	<0.0001	0.791	<0.0001
HPX	-0.624	0.012	0.147	0.586	-0.735	0.002	-0.565	0.025	-0.606	0.015	-0.615	0.013
HSPA4&	0.424	0.296	0.496	0.212	0.335	0.417	0.081	0.848	0.743	0.035	0.531	0.176
HSPA5	-0.771	0.002	0.13	0.66	-0.842	<0.0001	-0.798	0.001	-0.648	0.014	-0.613	0.022
HYDIN	-0.495	0.075	0.292	0.31	-0.71	0.006	-0.688	0.008	-0.552	0.044	-0.411	0.146
ICA	-0.433	0.124	0.609	0.024	-0.618	0.021	-0.503	0.069	-0.486	0.081	-0.459	0.101
IGH-1A	-0.714	0.004	0.064	0.823	-0.686	0.006	-0.675	0.007	-0.55	0.036	-0.507	0.056
IGHE₽	0.498	0.119	-0.296	0.378	0.36	0.278	0.282	0.4	0.722	0.012	0.619	0.043
IGHG1	-0.841	0.001	0.441	0.089	-0.637	0.022	-0.574	0.022	-0.7	0.003	-0.692	0.011
IGHV1-18	0.697	0.007	0.648	0.014	0.591	0.029	0.644	0.015	0.543	0.048	0.499	0.072
IGHV1-5	-0.51	0.132	0.154	0.67	-0.275	0.442	-0.402	0.249	-0.669	0.034	-0.655	0.04
IGHV3-1	-0.701	0.007	0.2	0.492	-0.728	0.004	-0.697	0.007	-0.591	0.029	-0.53	0.054
IGHV4-1	0.29	0.275	0.528	0.036	0.061	0.822	-0.043	0.873	0.333	0.208	0.266	0.319
IGHV6-3*	0.144	0.785	0.854	0.031	0.102	0.847	0.437	0.386	-0.477	0.338	0.093	0.861
IGHV7-1	-0.248	0.391	0.486	0.081	-0.569	0.037	-0.591	0.029	-0.407	0.151	-0.24	0.409
IGKV14-126	-0.659	0.038	-0.162	0.656	-0.483	0.157	-0.54	0.107	-0.559	0.093	-0.637	0.048
IGKV3-7	0.161	0.567	0.621	0.016	-0.032	0.913	0.05	0.863	-0.046	0.873	0	>0.999
IGKV4-63	-0.721	0.023	0.055	0.892	-0.685	0.035	-0.649	0.049	-0.539	0.114	-0.552	0.105
IGKV6-13	0.473	0.088	-0.218	0.453	0.511	0.062	0.557	0.039	0.374	0.188	0.386	0.173
IGKV7-33Ł	0.864	0.059	0.924	0.025	0.361	0.55	0.583	0.303	-0.487	0.406	-0.278	0.651
IGKV8-27	0.601	0.043	-0.14	0.667	0.378	0.228	0.252	0.43	0.587	0.049	0.469	0.128
ITIH1	0.635	0.01	0.374	0.155	0.524	0.04	0.685	0.004	0.382	0.145	0.338	0.2
ITIH2	0.935	<0.0001	0.206	0.443	0.756	0.001	0.729	0.002	0.809	<0.0001	0.844	<0.0001
ITIH3	-0.811	<0.0001	0.05	0.863	-0.643	0.012	-0.629	0.014	-0.796	0.001	-0.739	0.002
ITIH4	-0.612	0.014	0.185	0.725	-0.653	0.007	-0.503	0.049	-0.609	0.014	-0.594	0.017
KLKB1	0.891	<0.0001	0.059	0.831	0.788	0.001	0.815	<0.0001	0.788	0.001	0.797	<0.0001
KNG2	-0.518	0.042	0.165	0.541	-0.697	0.004	-0.588	0.019	-0.424	0.104	-0.435	0.094
LIFR	0.87	<0.0001	0.132	0.64	0.789	0.001	0.786	0.001	0.919	>0.999	0.833	<0.0001
MASP1	0.586	0.017	0.417	0.109	0.512	0.043	0.517	0.04	0.665	0.005	0.537	0.032
MBL1&	0.537	0.17	0.174	0.68	0.821	0.012	0.573	0.138	-0.036	0.933	0.121	0.775
MUG1	0.879	<0.0001	0.082	0.763	0.797	<0.0001	0.791	<0.0001	0.747	0.001	0.735	0.002
MUP1&	0.403	0.322	0.781	0.022	0.479	0.23	0.329	0.426	-0.446	0.269	-0.676	0.066
NUCB1	0.398	0.127	0.563	0.023	0.156	0.565	0.028	0.917	0.356	0.176	0.329	0.214
P01645*	0.036	0.964	0.893	0.012	0.036	0.964	0.286	0.556	-0.036	0.964	0.214	0.662
P01801	-0.588	0.038	0.044	0.892	-0.676	0.014	-0.698	0.01	-0.637	0.022	-0.522	0.071
PGLYRP2	-0.719	0.005	0.139	0.638	-0.811	0.001	-0.767	0.002	-0.534	0.052	-0.481	0.084
PKLR	0.603	0.038	0.123	0.704	0.661	0.019	0.594	0.042	0.591	0.043	0.645	0.024
PLA2G7	0.6	0.026	0.481	0.084	0.477	0.087	0.67	0.011	0.543	0.048	0.455	0.104
PLTP&	0.648	0.023	0.27	0.396	0.665	0.018	0.751	0.005	0.366	0.242	0.424	0.17
POSTN&	0.687	0.014	0.506	0.093	0.589	0.044	0.678	0.015	0.695	0.012	0.469	0.124
PROZ	-0.684	0.009	-0.007	0.988	-0.521	0.059	-0.569	0.037	-0.723	0.005	-0.648	0.014
PSAP&	0.822	0.002	0.497	0.12	0.686	0.02	0.569	0.068	0.571	0.067	0.591	0.056
SAA1	-0.871	<0.0001	-0.206	0.443	-0.638	0.009	-0.615	0.013	-0.747	0.001	-0.682	0.005
SAA2	-0.806	<0.0001	-0.282	0.289	-0.635	0.01	-0.553	0.029	-0.621	0.012	-0.618	0.013
SAA4	-0.724	0.002	0.109	0.689	-0.656	0.007	-0.638	0.009	-0.674	0.005	-0.727	0.002
SELL	0.327	0.327	-0.036	0.924	0.191	0.577	0.118	0.735	0.5	0.122	0.636	0.04
SERPINA10	-0.582	0.02	0.065	0.814	-0.638	0.009	-0.535	0.035	-0.477	0.064	-0.485	0.059
SERPINA3K	-0.471	0.068	0.409	0.117	-0.668	0.006	-0.541	0.033	-0.477	0.064	-0.471	0.068
SERPINA3N	-0.139	0.638	0.776	0.002	-0.402	0.155	-0.182	0.532	-0.191	0.512	-0.169	0.563
SERPINA7	-0.055	0.863	0.571	0.045	-0.165	0.591	-0.071	0.821	-0.093	0.765	-0.066	0.835
SERPINF2	-0.707	0.004	0.196	0.482	-0.754	0.002	-0.718	0.004	-0.525	0.047	-0.529	0.045
SERPING1	-0.543	0.03	-0.004	0.987	-0.543	0.03	-0.516	0.041	-0.236	0.379	-0.391	0.135

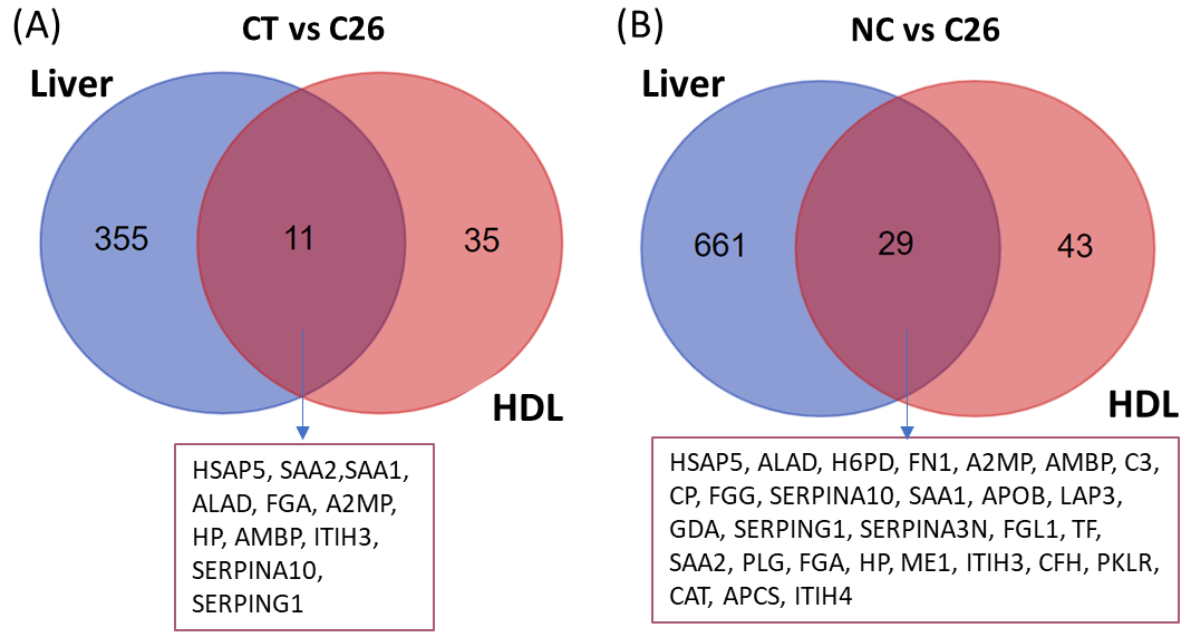
SOD3	-0.309	0.385	-0.76	0.011	-0.26	0.468	-0.523	0.121	0.107	0.769	-0.124	0.734
SPP2&	0.782	0.011	0.37	0.296	0.588	0.081	0.782	0.011	0.224	0.537	0.152	0.682
STIP1	-0.11	0.723	-0.593	0.036	0.055	0.863	-0.088	0.778	0.082	0.793	0.016	0.964
TF	-0.377	0.151	0.347	0.188	-0.588	0.019	-0.494	0.054	-0.35	0.184	-0.394	0.132
VPS52	-0.523	0.055	0.074	0.802	-0.445	0.111	-0.55	0.041	-0.555	0.039	-0.563	0.036
VTN	-0.774	0.001	-0.135	0.617	-0.735	0.002	-0.768	0.001	-0.603	0.015	-0.597	0.017
THBS4&	0.933	0.001	0.633	0.076	0.333	0.385	0.633	0.076	0.45	0.23	0.533	0.148
CDH13&	0.848	0.008	0.705	0.051	0.494	0.213	0.448	0.265	-0.365	0.374	-0.103	0.809
ACTR5	0.579	0.026	0.343	0.211	0.371	0.174	0.439	0.103	0.354	0.196	0.489	0.067



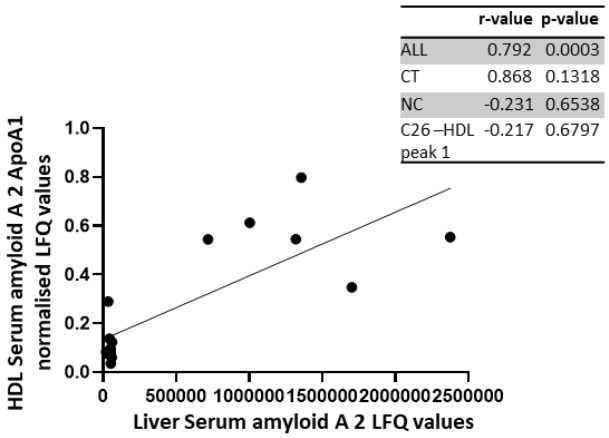
*Correlation analysis was carried out in GraphPad prism using the ApoA1 normalised LFQ values of proteins identified on the HDL proteome and %iBW Δ bodyweight, liver, gastrocnemius, tibialis, BAT and SAT . If an LFQ value was recorded as 0 for the HDL proteome then it was omitted from the analysis. A Pearson correlation was used for normally distributed data and a Spearman correlation for non-normally distributed data. &= majority of samples for analysis are in the CT and NC group, as protein was not identified in C26 group. *=majority of samples for analysis are in the C26 group, as protein was not identified in CT and NC groups. Ꝑ= majority of samples for analysis are in the NC and C26 group, as protein was not identified in CT group. ɬ = majority of samples for analysis are in the NC group, as protein was not identified in CT and C26 groups*

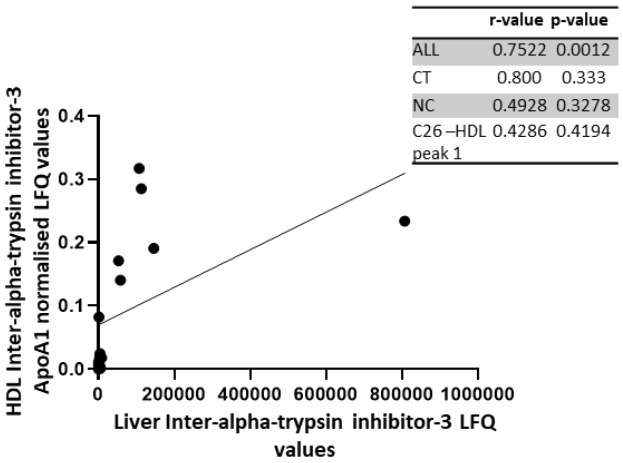
Select proteins identified as significant in the HDL proteome correlate with its expression in the liver:

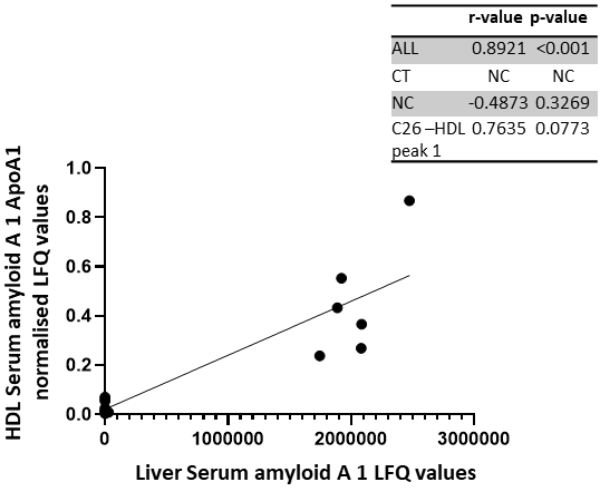
To determine if there was a relationship between proteins identified in the HDL proteome and liver, significant proteins identified in both the liver and HDL when comparing cachexia (C26-HDL peak 1 used for HDL analysis) to CT and NC were used for correlation analysis (Table 7). When comparing CT to C26, *n*=11 proteins were identified as significant in both the liver and HDL proteome (Figure 13A) including 78 kDa glucose-regulated protein (HSPA5), SAA2, SAA1, FGA, HP, ITIH3 and A2MP. When comparing NC to C26-HDL peak 1, *n*=29 proteins were identified as significant in both the liver and HDL proteome (Figure 13B), including HSPA5, delta-aminolevulinic acid dehydratase (ALAD), GDH/6PGL endoplasmic bifunctional protein (H6PD), FN1, A2MP, complement C3 (C3) and ceruloplasmin (CP). LFQ values of proteins in the liver and ApoA1 normalised HDL LFQ values were then correlated for each protein. Out of *n*=29 proteins collectively identified in the liver and HDL proteome when comparing C26 to CT and NC, *n*=14 were significant correlated when all groups were used in the analysis, HSAP5 (r=0.6044, p<0.05), AMBP (r=0.6755, p<0.01), SERPINA10 (r=0.6794, p<0.01), SAA2 (r=0.792, p<0.001), ITIH3 (r=0.7522, p<0.05), FN1 (r=0.5176, p<0.05), C3 (r=0.5059, p<0.05), CP (r=0.5471, p<0.05), SAA1 (r=0.8921, p<0.001), APOB (r=0.5971, p<0.05), plasminogen (PLG) (r=0.7536, p<0.01), PKLR (r=0.6701, p<0.05), APCS (r=0.6324, p<0.05), and ITIH4 (r=0.7353, p<0.01). Only *n*=1 protein was significantly correlated in an individual group (NC), FN1 (r=0.9429,p<0.05) (Table 7).

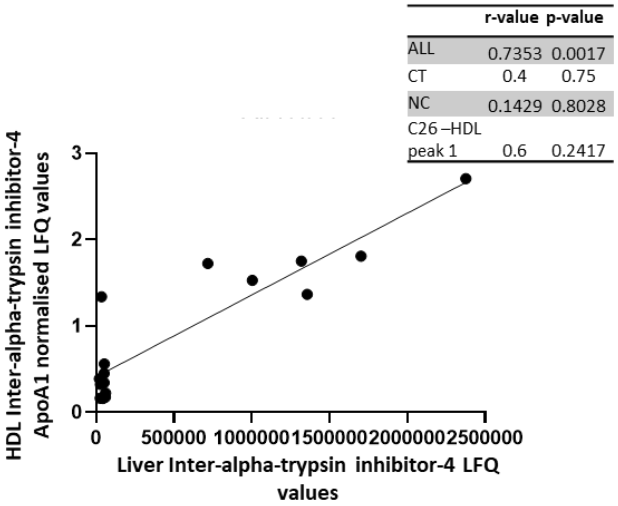

(A)
CT vs C26
Liver
355
11
35
HDL
HSAP5, SAA2,SAA1, ALAD, FGA, A2MP, HP, AMBP, ITIH3, SERPINA10, SERPING1
(B)
NC vs C26
Liver
661
29
43
HDL
HSAP5, ALAD, H6PD, FN1, A2MP, AMBP, C3, CP, FGG, SERPINA10, SAA1, APOB, LAP3, GDA, SERPING1, SERPINA3N, FGL1, TF, SAA2, PLG, FGA, HP, ME1, ITIH3, CFH, PKLR, CAT, APCS, ITIH4

(C)


r-value p-value
ALL 0.792 0.0003
CT 0.868 0.1318
NC -0.231 0.6538
C26 –HDL peak 1 -0.217 0.6797
HDL Serum amyloid A 2 ApoA1 normalised LFQ values
Liver Serum amyloid A 2 LFQ values


r-value p-value
ALL 0.7522 0.0012
CT 0.800 0.333
NC 0.4928 0.3278
C26 –HDL peak 1 0.4286 0.4194
HDL Inter-alpha-trypsin inhibitor-3 ApoA1 normalised LFQ values
Liver Inter-alpha-trypsin inhibitor-3 LFQ values


r-value p-value
ALL 0.8921 <0.001
CT NC NC
NC -0.4873 0.3269
C26 –HDL peak 1 0.7635 0.0773
HDL Serum amyloid A 1 ApoA1 normalised LFQ values
Liver Serum amyloid A 1 LFQ values


r-value p-value
ALL 0.7353 0.0017
CT 0.4 0.75
NC 0.1429 0.8028
C26 –HDL peak 1 0.6 0.2417
HDL Inter-alpha-trypsin inhibitor-4 ApoA1 normalised LFQ values
Liver Inter-alpha-trypsin inhibitor-4 LFQ values

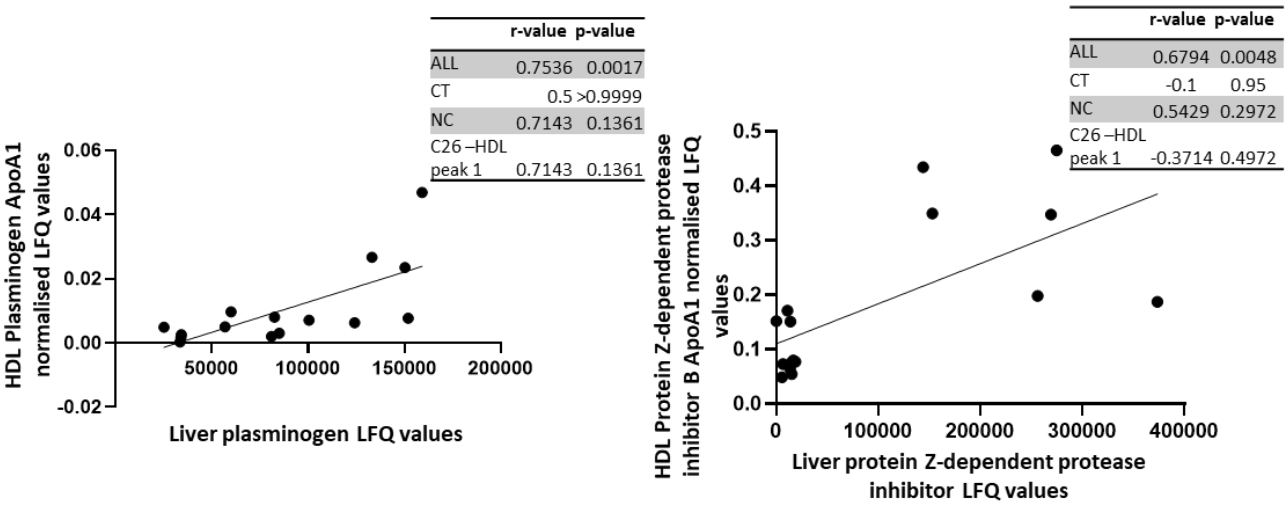
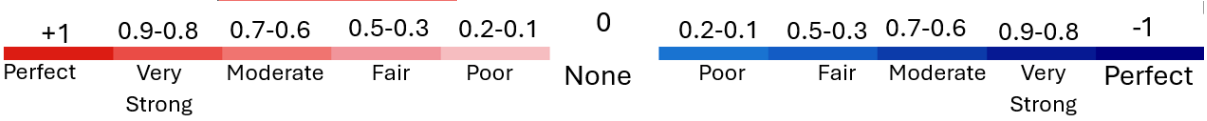


Figure 13: Venn diagrams of significant proteins identified in the liver and HDL of (A) CT versus C26-HDL peak 1 and (B) NC versus C26-HDL peak 2. (C) Correlation analysis of the proteins with the highest correlation r-value identified on both the liver and HDL of CT versus C26 or NC versus C26. Correlation analysis was carried out in GraphPad prism using the LFQ values of proteins identified in the liver and ApoA1 normalised LFQ values of proteins identified on the HDL proteome. If an LFQ value was recorded as 0 for the HDL proteome then it was omitted from the analysis. A Pearson correlation was used for normally distributed data and a Spearman correlation for non-normally distributed data.

Table 7: Proteins identified as significant in the liver and HDL proteome of C26 when compared to CT and NC mice.

	All		CT		NC		C26	
	r-value	p-value	r-value	p-value	r-value	p-value	r-value	p-value
HSAP5[a,b]	0.6044	0.0248	NC	NC	-0.1429	0.8028	0.0857	0.9194
ALAD[a,b]	0.25	0.5948	NC	NC	-0.0857	0.9194	NC	NC
A2MP[a,b]	0.5385	0.0611	NC	NC	-0.9	0.0833	0.8286	0.0583
AMBP[a,b]	0.6755	0.0052	-0.9487	0.1667	0.08571	0.9194	0.5429	0.2972
SERPINA10[a,b]	0.6794	0.0048	-0.1	0.95	0.5429	0.2972	-0.3714	0.4972
SERPING1[a,b]	0.4176	0.1089	-0.4	0.75	-0.1429	0.8028	0.8286	0.0583
SAA2[a,b]	0.792	0.0003	0.868	0.1318	-0.231	0.6538	-0.217	0.6797
FGA[a,b]	0.4912	0.0556	-0.8	0.3333	-0.3714	0.4972	0.4286	0.4194
HP[a,b]	0.4147	0.1116	0.6	0.4167	0.4857	0.3556	0.1429	0.8028
ITIH3[a,b]	0.7522	0.0012	0.8	0.333	0.4928	0.3278	0.4286	0.4194
H6PD[b]	0.3736	0.2095	NC	NC	0	>0.9999	0.7714	0.1028
FN1[b]	0.5176	0.0423	-0.8	0.3333	0.9429	0.0167	0.4857	0.3556
C3[b]	0.5059	0.0479	0.6	0.4167	-0.3714	0.4972	0.3714	0.4972
CP[b]	0.5471	0.0305	0	>0.999	-0.2	0.7139	-0.2	0.7139
FGG[b]	0.1559	0.5635	-0.8	0.3333	-0.3714	0.4972	-0.0857	0.9194
SAA1[b]	0.8921	<0.001	NC	NC	-0.4873	0.3269	0.7635	0.0773
APOB[b]	0.5971	0.0165	0.4	0.75	0.6	0.2417	0.4857	0.3556
LAP3[b]	-0.4992	0.2079	NC	NC	0.4643	0.3536	NC	NC
GDA[b]	0.1923	0.5293	NC	NC	0.2	0.7139	0.6	0.35
SERPINA3N[b]	0.2695	0.3099	-0.7379	0.3333	-0.1429	0.8028	-0.0286	>0.9999
FGL1[b]	-0.356	0.644	NC	NC	NC	NC	-0.356	0.644
TF[b]	0.1118	0.6806	-0.6	0.4167	-0.6571	0.175	-0.7714	0.1028
PLG[b]	0.7536	0.0017	0.5	>0.9999	0.7143	0.1361	0.7143	0.1361
ME1[b]	0.3571	0.3894	NC	NC	0.6	0.2417	NC	NC
CFH[b]	-0.1176	0.6645	0.2	0.9167	0.4286	0.4194	0.08571	0.9194

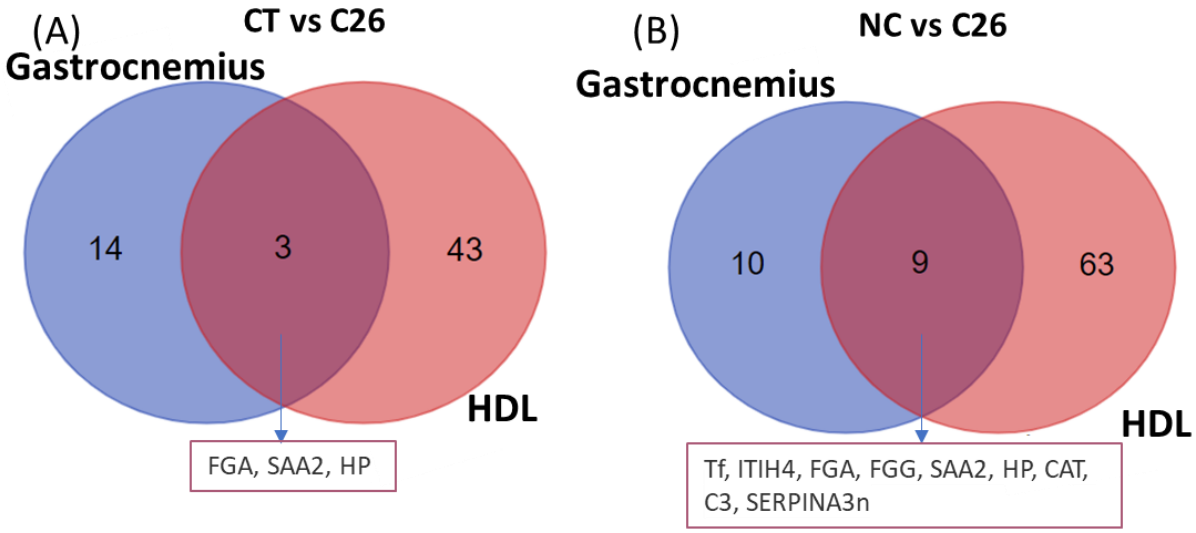
PKLR[b]	0.6701	0.0171	NC	NC	0.5555	0.2525	0.2671	0.7329
CAT[b]	-0.4294	0.0986	-0.4	0.75	0.4286	0.4194	-0.8286	0.0583
APCS[b]	0.6324	0.0101	-0.8	0.3333	-0.3714	0.4972	0.08571	0.9194
ITIH4[b]	0.7353	0.0017	0.4	0.75	0.1429	0.8028	0.6	0.2417



Correlation analysis was carried out in GraphPad prism using the LFQ values of proteins identified in the liver and ApoA1 normalised LFQ values of proteins identified on the HDL proteome. If an LFQ value was recorded as 0 for the HDL proteome then it was omitted from the analysis. A Pearson correlation was used for normally distributed data and a Spearman correlation for non-normally distributed data. [a]= proteins identified in the liver and HDL of CT vs C26. [b]= proteins identified in the liver and HDL of NC vs C26. NC= not calculated if it was not identified in enough samples to calculate result.

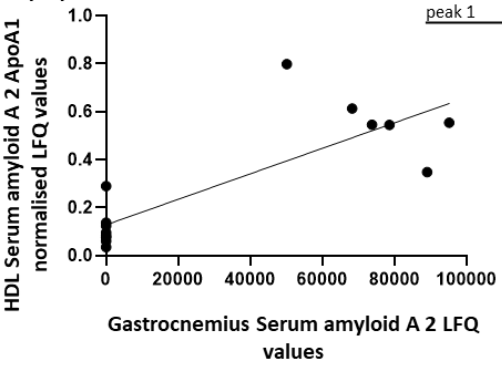
<u>Select proteins identified as significant in the HDL proteome correlate with its expression in the gastrocnemius:</u>

To determine if there was a relationship between proteins identified in the HDL proteome and gastrocnemius, significant proteins identified in both the gastrocnemius and HDL when comparing cachexia (C26-HDL peak 1 used for HDL analysis) to CT and NC were used for correlation analysis (Table 8). When comparing CT to C26, *n*=3 proteins were identified as significant on both the gastrocnemius and HDL (Figure 14A), FGA, SAA2 and HP. When comparing NC to C26, *n*=9 proteins were identified as significant on both the gastrocnemius and HDL (Figure 14B), TF, ITIH4, FGA, FGG, SAA2, HP, CAT, C3 and SERPINA3n. LFQ values of proteins in the gastrocnemius and ApoA1 normalised HDL LFQ values were then correlated for each protein. Out of *n*=9 proteins collectively identified in the gastrocnemius and HDL proteome when comparing C26 to CT and NC, *n*=5 were significantly correlated when all groups were used in the analysis, SAA2 ($r=0.7723$, $p<0.001$), C3 ($r=0.7382$, $p<0.01$), TF ($r=0.7324$, $p<0.01$), ITIH4 ($r=0.7324$, $p<0.01$) and CAT ($r=0.5235$, $p=0.0397$). There were no significantly correlated proteins when groups were individually analysed.

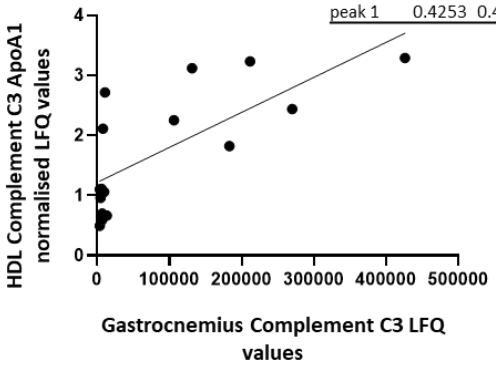


(C)

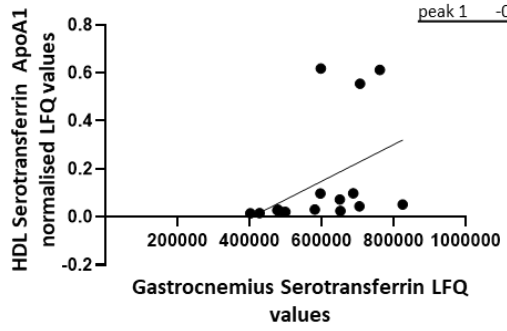
	r-value	p-value
ALL	0.7723	0.0007
CT	NC	NC
NC	NC	NC
C26 –HDL peak 1	-0.6571	0.175



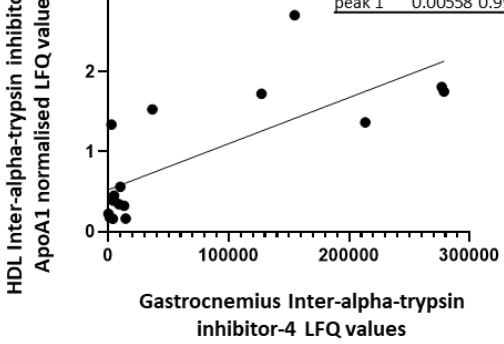
	r-value	p-value
ALL	0.7382	0.0016
CT	0.887	0.113
NC	-0.0943	0.859
C26 –HDL peak 1	0.4253	0.4005



	r-value	p-value
ALL	0.7324	0.0018
CT	0.8084	0.1916
NC	0.4897	0.3242
C26 –HDL peak 1	-0.2834	0.5863



	r-value	p-value
ALL	0.7439	0.001
CT	-0.738	0.262
NC	0.7668	0.0752
C26 –HDL peak 1	0.00558	0.9916



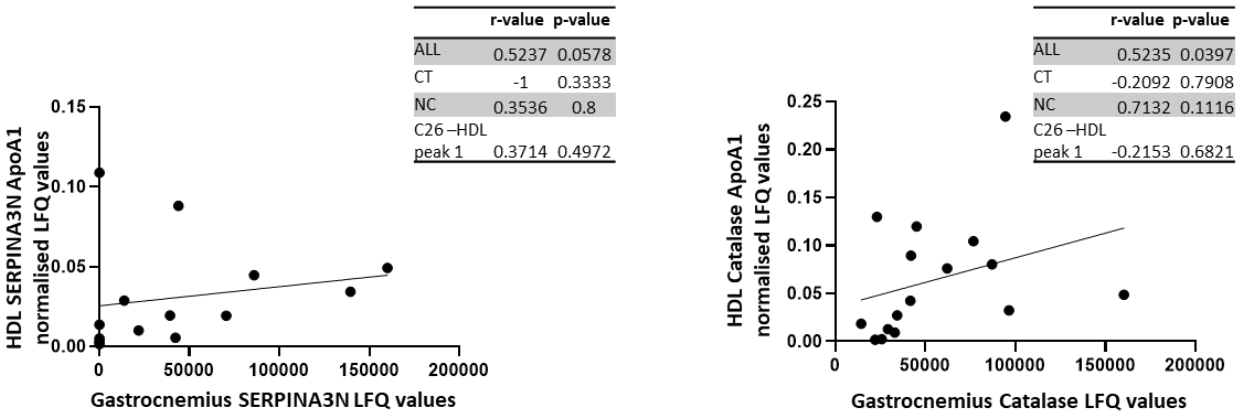
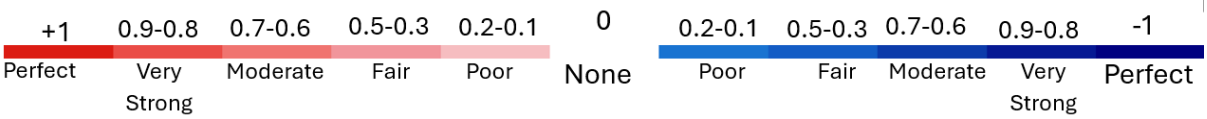


Figure 14: Venn diagrams of significant proteins identified in the gastrocnemius and HDL of (A) CT versus C26 and (B) NC versus C26. (C) Correlation analysis of the proteins with the highest correlation r-value identified on both the gastrocnemius and HDL of CT versus C26 or NC versus C26. **Correlation analysis was carried out in GraphPad prism using the LFQ values of proteins identified in the gastrocnemius and ApoA1 normalised LFQ values of proteins identified on the HDL proteome. If an LFQ value was recorded as 0 for the HDL proteome then it was omitted from the analysis. A Pearson correlation was used for normally distributed data and a Spearman correlation for non-normally distributed data.**

Table 8: Proteins identified as significant in the gastrocnemius and HDL proteome of C26 when compared to CT and NC mice.

	All		CT		NC		C26	
	r-value	p-value	r-value	p-value	r-value	p-value	r-value	p-value
TF[b]	0.7324	0.0018	0.8084	0.1916	0.4897	0.3242	-0.2834	0.5863
ITIH4[b]	0.7439	0.0010	-0.738	0.262	0.7668	0.0752	0.0056	0.9916
FGA[a,b]	0.1912	0.4769	-0.3832	0.6168	-0.6287	0.1812	-0.241	0.6455
FGG	0.1265	0.6405	-0.4807	0.5193	-0.3517	0.4942	-0.1158	0.827
SAA2[a,b]	0.7723	0.0007	NC	NC	NC	NC	-0.6571	0.175
HP[a,b]	0.2208	0.4082	-0.0303	0.9697	0.3877	0.4476	0.7057	0.1172
CAT[b]	0.5235	0.0397	-0.2092	0.7908	0.7132	0.1116	-0.2153	0.6821
C3[b]	0.7382	0.0016	0.887	0.113	-0.09425	0.859	0.4253	0.4005
SERPINA3N[b]	0.5237	0.0578	-1	0.3333	0.3536	0.8	0.3714	0.4972



Correlation analysis was carried out in GraphPad prism using the LFQ values of proteins identified in the gastrocnemius and ApoA1 normalised LFQ values of proteins identified on the HDL proteome. If an LFQ value was recorded as 0 for the HDL proteome then it was omitted from the analysis. A Pearson correlation was used for normally distributed data and a Spearman correlation for non-normally distributed data. [a]= proteins identified in the gastrocnemius and HDL of CT vs C26. [b]= proteins identified in the gastrocnemius and HDL of NC vs C26. NC= not calculated if it was not identified in enough samples to calculate result.

Discussion

The C26 mouse model of cachexia allowed the exploration of novel insights into potential relationships between the HDL and liver proteome in cancer cachexia. Our findings demonstrate significant alterations in the HDL proteome in cancer cachexia, that are not evident in tumour bearing mice without cachexia. Correlation analysis allowed us to further determine which HDL proteins had a strong relationship to adipose or muscle tissue, potentially highlighting proteins associated with or affected by the wasting process. This study also adds to the growing body of literature on the role of the liver in cachexia development, identifying potentially targetable pathways. Finally, this study allowed us to substantiate previous work in patient samples, wherein we utilised the HDL proteome to determine biomarkers of cachexia.

The altered distribution of LDL in both cancer models, NC and C26 mice, and HDL in C26 mice was unanticipated. The chromatogram of CT mice shows a typical FPLC isolation of LDL, HDL and albumin. However, there was a double peak in NC and C26 mice where there is typically one LDL peak and a double peak in C26 mice where there is typically one HDL peak. Proteomic analysis showed that ApoB was present in all "LDL" peaks except for NC-LDL peak 1, where it is absent from at least 70% of samples. This would suggest that LDL is not present in this peak or perhaps its identification is masked by other abundant proteins. ApoA1 is a unique apolipoprotein exclusive to HDL, it was present in both C26 HDL peaks, confirming the presence of the particle. HDL is typically downregulated in cachexia[658, 659, 667], with large HDL particularly affected in one study of C26 mice[659]. It is possible the double HDL peak we see in the C26 mice are due to extreme changes in size distribution, with the first peak representing large HDL and the second peak representing small HDL. This may be supported by the increase in scavenger receptor class B member 1 (SCARB), also known as SR-B1, in the liver of C26 mice when compared to CT mice. This receptor binds to ApoA1 and mediates the transfer of cholesterol from HDL[668]. SCARB1 knockout mice have large, cholesterol rich HDL[668], and while the effect of SCARB1 overexpression on HDL size has not been investigated (although it decreases circulating levels of HDL[669]) it may result in less cholesterol rich, smaller HDL. The presence of ApoA1, but not cholesterol in this peak (as shown by the cholesterol assay in supplementary figure 1) potentially supports this theory, however further research is needed.

In terms of HDL proteomic signatures of cancer cachexia, C26 HDL (C26-HDL peak 1) was enriched with acute phase proteins (APP) (SERPINA3n, CP, SAA2), exhibited reduced levels of metabolic enzymes (Guanine deaminase (GDA), pyruvate kinase (Pklr)) and had distinct changes in apolipoprotein content (ApoE, ApoD, ApoH), compared to HDL of CT and NC mice. The acute phase response (APR) is well documented in cancer cachexia[3, 179, 477, 646] and is a key feature of the C26 model. The APR is produced

in response to tumour or host derived inflammatory cytokines such as IL-6 and TNF-α[479] and while originally thought to be only liver derived, previous work in this model has shown that muscle is also a producer[335]. HDL becomes enriched with APP during inflammatory conditions[227, 660, 670], with SAA being one of the most well studied[353]. Whether SAA is just a marker of inflammation or negatively affects HDL is not clear. Studies suggest that SAA enriched HDL impairs cholesterol efflux capacity[301, 355] and anti-inflammatory function[354, 355] however it has also been shown to promote cholesterol efflux[671] and prevent HDL oxidation[359]. SAA1, SAA2 and SAA4 were upregulated on the HDL of C26 mice, compared to CT and NC mice, and negatively correlated with changes in bodyweight, gastrocnemius, tibialis, BAT and SAT (%iBW). Levels of SAA1 and SAA2 correlated with levels of the protein in the liver (r=0.8921 and 0.792 respectively) while SAA2 correlated with levels of the protein in the muscle (r=0.7723), potentially highlighting similar activation of the acute phase response in the two tissues. In a previous study with C26, BAF3 and KP53 mice, SAA1 mRNA, along with other APPs, was upregulated in gastrocnemius muscle and correlated with the marker of degradation, atrogin-1[335]. SAA has a direct effect on C2C12 myotubes, inducing atrogin-1 expression and reducing myotube size by 10-13%[672]. These effects were mediated through toll like receptor (TLR)-2 which stimulated the production of IL-6 and TNF-α[672]. Conversely, tumour derived SAA in Lewis lung carcinoma mice did not signal through TLR-2 even in the presence of muscle wasting[673].

The acute phase protein SERPINA3n was upregulated in the HDL of C26 mice compared to NC, but not CT, mice. Additionally, SERPINA3n was elevated in the muscle of C26 mice relative to NC mice, and in the liver when compared to both NC and CT mice. HDL SERPINA3n did not correlate with levels in the liver but almost correlated with levels in the muscle (r=0.5237, p=0.0578), suggesting a stronger relationship with muscle acute phase activation rather than liver. Interestingly, SERPINA3n correlated with liver weight (%iBW) but not muscle, adipose or change in bodyweight, suggesting it is not a marker of muscle atrophy or fat loss, but potentially a reflection of inflammation. Hulmi et al. showed that SERPINA3n was upregulated in the liver, muscle and serum of C26 mice, and this was reflective of the inflammatory state (as measured by spleen mass and IL-6) mores so than muscle wasting[485]. Massart et al., also found that muscle mRNA SERPINA3 (the human equivalent) positively correlated with C-Reactive Protein (CRP), a measure of inflammation, and negatively correlated with skeletal muscle index, despite the protein being cancer but not cachexia specific[335]. SERPINA3n has been identified in the muscle of mice in several studies[335, 646, 674], but it has not been well investigated in circulation. Therefore, using HDL as a non-invasive way to measure SERPINA3n, which may reflect changes in the muscle, is a promising development[335, 646, 674].

CP was also increased on the HDL of C26 mice compared to NC and in the liver compared to NC and CT mice. Levels of HDL CP correlated with its concentration in the liver ($r=0.5471$, $p<0.05$), and negatively correlated with gastrocnemius, tibialis, BAT and SAT (%iBW). CP is a liver derived copper carrying protein with both pro- and anti-oxidant properties[675]. CP accumulates on HDL during the APR[618], impairing its ability to protect LDL from oxidation and inhibit the expression of monocyte chemoattractant protein-1 (MCP-1)[227, 676]. CP is a potential biomarker of metabolic dysregulation. Elevated serum levels of the protein were found in diabetes[308], obesity[677] and metabolic syndrome[678] and were particularly associated with elevated triglycerides and insulin resistance[677, 678], features that typify cachexia[679-681]. Elevated CP has also been identified in several cancer types[682-684] and was identified as a prognostic marker in glioma[685] and cervical cancer[686].

Several enzymes were also identified on HDL including GDA, PKLR, Delta-aminolevulinic acid dehydratase (ALAD) and biotinidase (BTD). Interestingly, the majority of these enzymes, excluding BTD and ALAD, where only significantly affected when comparing C26 and NC mice. However, when comparing NC and CT mice, only PKLR was significantly different between the two groups. This may suggest a progressive disturbance in metabolic pathways that are initiated with cancer but exacerbated in cancer cachexia. GDA and xanthine dehydrogenase (XDH) (or xanthine oxidase) are both involved in purine metabolism[687] and were downregulated on the HDL of C26 mice compared to NC. GDA was also downregulated in the livers of C26 mice compared to CT and NC mice, however XDH was not affected. The purine catabolism pathway converts purine base pairs adenosine and guanosine into uric acid[688]. GDA converts guanine into xanthine, while XDH converts xanthine into uric acid[688]. Contradictory to our results, the purine pathway is upregulated in some studies of cancer and cachexia. Xanthine oxidase activity was upregulated in the liver[689, 690], serum[690] and brain of tumour-bearing rats[689, 691] while uric acid was upregulated in tumour-bearing rats[689, 690] and hepatocellular carcinoma (HCC) patients[690]. Xanthine, a product of GDA activity, was upregulated in HCC bearing rats[690] and XDH mRNA was upregulated in the liver of HCC rats[690], further promoting activation of the this pathway in cancer. Interestingly, one study in C26 mice showed no changes in uric acid and a “non-significant decrease” in xanthine oxidase ($p=0.068$) in tumour bearing mice relative to controls[692]. This shows the activation of the purine catabolism pathway in cachexia may be model specific and is actually reduced in the C26 model.

PKLR and malic enzyme (ME1) were also downregulated on the HDL (and livers) of C26 mice, in comparison to NC mice. ME1 converts malate into pyruvate[693] while PKLR converts phosphoenolpyruvate into pyruvate[694] which is then converted into acetyl-CoA for the TCA cycle[694], a pathway which is downregulated in the livers of the C26 mice. HDL PKLR correlated with concentrations

in the liver and were associated with the change in bodyweight, gastrocnemius, tibialis, BAT and SAT. However ME1 did not correlate with any body composition measure. Downregulation of PKLR in the liver was previously observed in an $Apc^{min/+}$ mouse model of cachexia, along with other glycolytic enzymes such as SLC2A2[695], which was similarly downregulated in the livers of C26 mice in this study compared to NC mice. This suggest that hepatic glycolysis is dysregulated in cachexia, a conclusion supported by earlier research in C26 mice[462, 463]. ME1 has not been well studied in cachexia, however, reduced levels of the protein have been found in adipose tissue of rats with cachexia[696].

Proteins of interest for their documented association with cachexia include IGFBP3 and LIFR. IGFBP3, an IGF-1 binding protein[697] normally produced by the liver[698], has been identified as a tumour secreted, pro-cachectic factor[63]. IGFBP3 is secreted by Capan-1 pancreatic and C26 colorectal cells and elevated serum levels were identified as a biomarker of cachexia in colorectal cancer patients[63, 699]. Wang et al., showed that tumour derived IGFBP3 disrupts IGF/insulin signalling to promote lipolysis and inhibit lipogenesis in adipocytes[63] while an IGFBP3 homolog in a drosophila cachexia model caused adipose loss[63]. Host derived IGFBP3 can also contribute to cachexia. NOTCH1 signalling induced IGFBP3 production in adipose tissue endothelial cells, causing adipose tissue loss by apoptosis and inhibition of adipogenesis[700]. IGFBP3 may also contribute to muscle loss in cachexia by inhibiting myogenesis and promoting protein degradation via dysregulation of PI3K/AKT signalling, as was found in C2C12 cells[699]. Additionally, mRNA of IGFBP3 was upregulated in the muscle of mice with pancreatic cancer associated muscle wasting[701]. Interestingly, IGFBP3 was downregulated on the HDL of C26 mice in comparison to NC mice. The increase of IGFBP3 on NC HDL could represent a fragment of the protein as a consequence of increased proteolysis, rather than an increase in total IGFBP3. In a small study of lung cancer patients, increased proteolysis of IGFBP3 was associated with less weight loss than the patients with intact IGFBP3[702]. Notably, IGFBP3 was upregulated on C26-HDL peak 2 in comparison to C26-HDL peak 1. If our theory of a distinct small HDL subset is valid, this could highlight a preference of IGFBP3 for small HDL particles compared to large HDL.

LIFR is a receptor for leukaemia inhibitory factor (LIF), an IL-6 cytokine family member that has been identified as a secreted cachexia inducing factor in many cancers[703], including the C26 model of cachexia[704]. Tumour secreted LIF induces adipose tissue loss by inhibiting hepatic lipogenesis[705], inducing lipolysis[706, 707] and reducing food intake[705, 707] while tumour[708] and host[709] derived LIF induced muscle wasting. LIFR dimerizes with gp130 and signals though through a number of pathways including JAK/STAT, ERK1/2 and p38 MAPK[703]. Liver specific knockout of LIFR was shown to attenuate cachexia, with reduced weight loss and increased survival in mice[705]. Interestingly, LIFR was not identified in the livers of mice in this study but it was decreased on the HDL of C26 mice when compared to CT and NC

mice and it positively correlated with Δ bodyweight, gastrocnemius, tibialis, BAT and SAT. This could indicate increased availability of the receptor for uptake by HDL as it is not being utilised in mice without cachexia or, potentially, a decrease in LIFR in an effort to reduce LIF signalling in C26 mice.

C26-HDL peak 2 is proteomically distinct from C26-HDL peak 1, including greater protein diversity, potentially supporting the presence of unique small HDL particle[710] in C26 mice. C26-HDL peak 2 is characterised by increases in acute phase proteins (ITIH4[711], ORM1[712], CP[227]), complement (VTN,C9,C6,C2) and tissue injury (ACTB, ACTG1[373]). While this is a novel and interesting find, it is important to note that we did not see a similar secondary HDL peak in patients with cachexia (unpublished data) and so this may be a unique feature of cachexia in C26 mice. There are differences between mouse and human HDL that could contribute to this discrepancy. For example, mice lack cholesteryl ester transfer protein (CETP) and carry the majority of their cholesterol on HDL particles while humans carry the majority on LDL[713]. HDL in mice also has less variation in particle size compared to humans[714], however, the distribution of proteins across HDL size and overall protein cargo was similar between the two species[713]. Further investigation is needed to determine the relevance and implications of these unique HDL particles in the C26 mouse model of cachexia.

The animal model allowed investigation of the effects of cachexia on hepatic pathways, wherein those related to protein synthesis, xenobiotic metabolism, and energy production were affected by cancer cachexia. Hepatic protein synthesis was upregulated including many pathways regulating the initiation of protein synthesis such as eukaryotic translation initiation, elongation and termination and eukaryotic initiation factor-2 (EIF2) signalling, which involves the processing of RNA into proteins via the ribosome. Indeed, many of the proteins identified in these pathways include ribosomal subunits such as 60S ribosomal protein L27a (RPL27a), 60S ribosomal protein L7 (RPL7) and 40S ribosomal protein SA (RPSA). Upregulation of hepatic protein synthesis in cachexia in C26 mice may be model specific. Indeed increased protein synthesis as measured by puromycin incorporation and increased phosphorylation of ribosomal S6 (indicating increased mTOR signalling) has been shown in C26 mice[638]. Samuels et al., credited increased protein synthesis in C26 mice with increased RNA content in the liver and therefore increased "capacity"[715]. Similar to our results, Khamoui et al., identified increased RNA processing, transport, binding and translation to be some of the upregulated pathways in the liver of C26 mice, indicating increased protein synthesis[634]. An $APC^{min/+}$ mouse model of cachexia[639] and a tumour-bearing rat model of cachexia also showed increased protein synthesis[650]. However, several models do not have increased hepatic protein synthesis[716-718] including a Lewis lung carcinoma model[717] and a MAC-16 colorectal model of cachexia[718]. Few studies investigated hepatic protein synthesis in patients with cachexia, presumably due to the challenges of obtaining liver samples (which HDL proteomics can

circumvent). Additionally, the existing studies have very small sample sizes. One study in pancreatic cancer patients (*n*=16) did show some indication of increased protein synthesis in patients with cachexia, such as increased AKT phosphorylation (+73%) and mTOR (+50%), however, these were not significant (p=0.079 and 0.113, respectively), potentially due to the small sample size[719]. Another study (*n*=31) showed decreased protein synthesis in weight losing cancer patients (gastric and colorectal), compared to weight stable patients[720]. Increased protein synthesis is thought to largely contribute to the acute phase response, with or without a decrease in structural proteins[462], as shown by decreased liver size[715]. As the C26 livers are not reduced in our study, we can assume the increased acute phase response (which is identified as an upregulated pathway) did not cause a reduction in structural proteins. The APR is stimulated by inflammation and amino acids released from muscle wasting[462]. Interestingly, response of eif2ak4 (gcn2) to amino acid deficiency is upregulated in the livers of C26 mice, suggesting that the amino acids derived from muscle may not be enough to keep up with demand. It is suggested that 2.6g of muscle protein is needed to synthesize 1g of the APP fibrinogen[721]. The APR may increase resting energy expenditure, as was found in pancreatic cancer patients with cachexia[179]. Hepatic protein synthesis can also increase energy expenditure through the production of protein synthesis machinery such as ribosomes[722]. The increased ribosomal proteins in our study suggest an increase in ribosomal biogenesis, similar to results found by Kojima et al., in the livers of mice with cachexia[101]. Hepatic xenobiotic metabolism (XNB) was also attenuated in the C26 mice, in comparison to CT and NC mice. The pathways identified include XNB signalling, XNB PXR signalling, XNB CAR signalling and XNB AHR signalling. Proteins identified in these pathways include members of the cytochrome P450 (CYP) enzymes such as CYP2D9, CYP2A5 and CYP2D10 which are involved in phase I of XNB and enzymes belonging to phase II XNB such as glutathione S-transferases, Gsta3 and Gstt1, UDP-glucuronosyltransferases, Ugt1a9 and Ugt2a3 and Sulfotransferases, Sult1d1 and Sult5a1. XNB is the conversion of toxic substances, including drugs, chemicals or metabolites, into hydrophilic molecules that can be excreted, protecting the body from their harmful effects[723]. Patients with cancer cachexia are at greater risk of treatment toxicity[24] which may be a worsened by XNB dysfunction in the liver. Downregulation of CYP3A activity, which metabolises ~60% of known drugs including anti-cancer treatments[724], was identified in several cachexia studies. CYP3A variants were downregulated in C26 mice in this study including CYP3A11, and CYP3A25, with the exception of CYP3A13. Rivory et al., showed that CYP3A drug metabolism was downregulated in cancer patients with an APR[724], increasing the risk of treatment toxicity. In another study, cachexia reduced the levels of CYP3A, resulting in an increase of plasma oxycodone and oxymorphone, administered for pain relief[725]. Similarly, CYP2D26 activity was reduced in head and neck cancer patients with cachexia, resulting in increased plasma tramadol levels (a pain relieving drug) and incidence of adverse effects affecting the central nervous

system such as drowsiness and delirium[726]. A study in rats showed that both CYP3A4 and CYP2D6 were reduced in cachexia and resulted in decreased clearance of the drugs midazolam and propranolo[727]. Dolly et al. also showed that CYP1A1 and CYP1A2, markers of AHR activation were reduced in C26, BAF3, MC38 and APC$^{min/+}$ mice, showing disturbances in XNB may not be model specific[728]. Feng et al., also found reductions in CYP enzymes and phase II enzymes in the livers of C26 mice which they attributed to disruptions in bile acid metabolism[729].

Thibaut et al.,[662] demonstrated that bile acid metabolism and bile acid flow are disrupted in C26 mice. Therefore, alterations in xenobiotic metabolism could be a reflection of changes in bile acid metabolism as these pathways are very interconnected. Finally, glucuronic acid, used in phase II XNB as a way to increase the hydrophilicity of substances, was upregulated in serum of patients with cachexia, potentially reflecting decreases in glycosyltransferases[606], as seen in this study. These findings underscore the complexity of XNB dysregulation in cachexia and its potential implications for drug metabolism and toxicity in affected patients.

Proteomics was also conducted on the gastrocnemius of the mice, however we identified an uncharacteristically low number of proteins from this tissue type. This is most likely due to using whole muscle wherein more abundant proteins may have masked the detection of less abundant ones. This is supported by previous research in these C26 mice which used the sarcoplasmic (SF) and myofibrillar fractions (MF) and identified a higher number of proteins[335]. Using this technique, Massart et al., identified 958 and 930 proteins respectively in the SF and MF, 228 of which were significantly different in the SF and 196 in the MF of C26 mice when compared to CT mice[335]. Nevertheless, the acute phase response was the most upregulated pathway in C26 mice identified by both this study and the study conducted using the MF and SF[335].

Finally, this study allowed us to substantiate potential biomarkers of cachexia and sarcopenia (low muscle mass) identified in a cohort of gastrointestinal cancer patients (chapter 3). In the original human study, patients were characterised based on the Fearon et al.,[2] definition of cachexia and Martin et al.,[465] definition of sarcopenia and a score was created for each condition with a high degree of sensitivity. While there were a number of differences between the human and mouse HDL proteome, such as the high number of proteins identified in mouse samples, and the identification of metabolic enzymes, some proteins appeared on both. The cachexia score in the human study had an area under the curve (AUC) of 0.810. This score shared the proteins ApoB, CP, HGFAC, PROC and SERPINA3n with C26-HDL peak 1 and VTN, ApoA2 and SERPINA7 with C26-HDL peak 2 (when compared to C26-HDL peak 1). HGFAC, CP and SERPINA3n were affected in the same way on human and mouse HDL, for example CP was upregulated in both, while PROC and ApoB were differently affected, for example ApoB

was downregulated on human HDL but upregulated on mouse HDL. C26-HDL peak 2 was not compared to CT HDL and so we cannot make direct comparisons in terms of significance with human HDL. The sarcopenia score in the human study had an AUC of 0.861. This score shared CP, HSPA5, IGFBP3, PROC and SERPINA3n with C26-HDL peak 1 and F2, CP, A2M, IGFBP3, ApoA2 and SERPINA7 with C26- HDL peak 2. All protein shared with C26-HDL peak 1 were similarly upregulated on human and mouse HDL during cachexia, except for PROC which was upregulated in humans and downregulated in mice.

In conclusion, this study is the first to demonstrate in mice that the HDL proteome is modulated by cancer cachexia, highlighting its biomarker potential. We have also correlated HDL remodelling with liver protein levels, showing how the HDL proteome can reflect adverse liver changes. Furthermore, our research contributes valuable insights into the liver's role in the development of cachexia, identifying pathways that may drive increased energy expenditure, such as protein synthesis, and those that may heighten patients' risk of adverse effects, such as xenobiotic metabolism. These findings pave the way for future biomarker development in cachexia, while also highlighting potential avenues for therapeutic intervention.

Supplementary material

Supplementary table 1: Proteins significantly different in the livers of CT versus C26 mice

Proteins	CT-Mean of LFQ values	C26- Mean of LFQ values	q-value
Aspg	70399	155802	0.03055
Mup9	7283975	1454736	0.01069
Mup10	2564275	824155	0.02123
Itih4	26548.75	1436120	<0.00001
Mup15	864340	24685	0.00105
Tmed7	157897.5	298521.3	0.01748
Serpina3i	3218.25	56286.63	0.0459
Fga	351935	2636750	<0.00001
Gcn1l1	87243.25	176480	0.02532
ApoB	141852.5	224691.3	0.04536
Mup7	626695	51600.91	0.0065
Aox3	677277.5	223036.3	0.00715
Kng1	94106.75	414772.5	0.00253
Ngp	542.8	79978	0.00564
Dld	1241300	864806.3	0.02867
Prkcsh	270650	523840	0.00057
Prdx4	3078800	4325500	0.01914
Flot1	10246.75	42517.75	0.015
Bhmt	7834400	10941463	0.04606
Calu	121615.5	306761.3	0.01269
Gamt	483695	311285	0.04477
Atp2a2	135627.3	213453.8	0.04405
Nnmt	4327.75	226241.6	0.01054
Wdr1	209970	334802.5	0.0438
Papss2	294262.5	125364.3	0.01202
Rdh7	2602700	1754488	0.00453
Ddc	194420	91465.38	0.01821
Cyp8b1	217932.5	32821.25	0.01963
Lgmn	81300.75	178421.3	0.01313
Rbm3	53673.75	156262.8	0.02122
Cyp1a2	1195128	223861.6	0.01496
C3	236595	1389813	<0.00001
C4b	145775	449170	0.00014
Ighm	50096.75	220749.9	0.04511
Cfb	150032.5	252357.5	0.01717
Mup3	948097.5	109128.3	0.01258
Saa1	0	2039488	<0.00001
Saa2	0	1511625	<0.00001
Ldha	2567275	4157013	0.0034
Ctsl	36505	91070.5	0.03406
Me1	1345050	713102.5	0.00594
Cfh	7814.425	242726.3	0.00015
Ttr	963910	130229.3	0.00058

Orm2	0	518508.8	<0.00001
Pdia4	638450	1499725	0.00256
Fcgr2	5775.475	68339.25	0.02046
Hsp90b1	1690625	3198213	0.00456
P4hb	3522100	5945000	0.00372
Alad	802160	561676.3	0.00268
Gstm1	18973250	9295538	0.00194
Fn1	318075	937852.5	0.00085
Mup2	357010	95253.88	0.00637
Cyp2d9	565550	283308.8	0.01513
Apcs	4276.675	6721688	<0.00001
Fabp1	19768750	12258188	0.03184
Umps	106105.8	171338.8	0.01485
Scd1	69063.75	3862.125	0.01829
Gsta1	57500.25	13974.51	0.04004
Rpl7	1262533	1790013	0.0318
Mdh1	6599725	5056363	0.01259
Calr	1472000	2749013	0.00889
Ca3	34662750	13971625	0.0006
Pah	1854900	435713.8	0.00341
Ass1	8380425	5630150	0.0036
Gapdh	10896075	8589138	0.01703
Eno1	3468100	2534413	0.01715
Ugt2b17	1018860	537742.5	0.01198
Cfl1	840630	1110766	0.03425
Fasn	2541600	986715	0.0059
Gstp1	31157750	17495500	0.02692
Gstm3	408067.5	215285	0.00363
Hspa5	3827725	6732875	0.00363
Plg	52593.75	139260	0.00482
Tgm2	354477.5	836512.5	0.00245
Ces1c	390527.5	180432.5	0.00389
Cat	7835125	2579025	0.00377
Ppib	933670	1492225	0.01283
Cyp2d10	1444325	810233.8	0.00257
Uox	4115300	1328326	0.00015
Hsd3b3	352067.5	109547.6	0.00035
S100a8	6374.55	533161.3	<0.00001
Man2a1	75251.25	254245	0.00258
Pdia3	2086050	3403013	0.00708
Pabpc1	744330	1092923	0.00646
Vtn	89991	291078.8	0.00107
Gsta3	6733525	3960375	0.00795
S100a9	22439.5	863938.8	0.00028
Scp2	6963350	4121013	0.00771
Wars	28359.5	131681.8	0.01723
Cyp2f2	1830475	606073.8	0.02533

Ephx2	2327275	1171470	0.00272
Pdha1	529455	270336.3	0.03485
G6pc	178667.5	106595.3	0.025
Rpl18	477707.5	709310	0.04614
Sar1a	74002.5	134621.4	0.03399
Col18a1	46514.75	106961	0.00249
Inmt	2774625	1138914	0.00369
Acsl1	2781875	1213308	0.00017
Stat3	40985.25	217912.5	<0.00001
Plin2	105239.8	289198.8	0.02562
Fkbp2	171922.3	306428.8	0.04602
Stt3a	273475	430795	0.03391
Aldh2	7994850	5017938	0.00883
Aldh3a2	268302.5	92513.75	0.0091
Rplp1	3287250	4920963	0.02207
Rpl36	122085.3	326102.5	0.03843
Eif1	315810	512390	0.01495
Hnrnpa1	54071.75	214418.8	0.01209
Ahcy	5319075	4166900	0.02282
Fmo1	564945	187592.5	0.00276
Shmt1	1931350	1363613	0.023
Atp6v1a	28046.5	79077.38	0.02535
Hsd17b4	1113168	784053.8	0.03094
Pkm	90156.5	267590	0.00389
Gck	116681.8	56095.13	0.0459
Rpl10a	802495	1238500	0.01566
Dbt	574095	397593.8	0.02764
Pklr	1431925	640072.5	0.00338
St3gal1	0	53057.75	0.00052
Abcd3	534087.5	259483.8	0.00063
Erp29	245610	415175	0.04789
Tm9sf2	12814	45556.75	0.01972
Idi1	199830	330835	0.03174
Eef2	1603900	2381825	0.00381
Gulo	917007.5	539240	0.03011
Eif3e	60661.75	104869.8	0.00594
Eif4a1	455840	684733.8	0.02226
Rpl26	332880	512303.8	0.04441
Abat	1031768	772830	0.01902
Copz1	54582.5	118622.3	0.00782
Rpl18a	424072.5	644025	0.04694
Rpl23a	305062.5	611692.5	0.00515
Rps28	477792.5	720103.8	0.04478
Rpl8	653395	976147.5	0.01733
Tpt1	389402.5	566890	0.01909
Rps12	543317.5	872391.3	0.00797
Gnb2l1	838510	1295450	0.01504

Akr1c6	10711025	6118413	0.02953
Ap2m1	102417.3	196293.8	0.03392
Serping1	29115.08	229905	0.00226
Anpep	137157.5	312065	0.00333
Gclc	649680	387262.5	0.00259
Ctsc	12055.25	133598	0.00133
Fmo5	1877225	1124823	0.00488
Tubb5	310895	416225	0.03288
Egfr	335130	721083.8	0.00252
Nucb1	139435	283162.5	0.00599
Rac2	4911.5	67018.5	0.02684
Fabp5	235075	1403230	<0.00001
Pc	3156725	4220463	0.03772
Clu	50263	262145	0.00385
Ambp	16190.75	392166.3	0.00026
Ugt2b36	6016400	3499000	0.02237
Mccc2	439522.5	341802.5	0.04786
Sec31a	342962.5	527997.5	0.01388
Sult1d1	344472.5	223767.5	0.01589
Slc27a5	775677.5	1004541	0.04669
Cyp2c67	651187.5	184178.8	0.01926
Gls2	529365	282250	0.00329
Acbd5	203252.5	89920.88	0.02047
Arcn1	246342.5	397363.8	0.0127
Orm1	37931.75	1035866	<0.00001
Myl6	490445	708117.5	0.02367
Grb2	14066.75	54017.5	0.00386
Serpinb6	59143	100166.1	0.01202
Pter	243097.5	402298.8	0.00343
Adck3	402350	74591.63	0.00033
Scarb1	303920	404956.3	0.03539
Cp	68379.25	214351.3	0.00511
Serpinf2	126690	345667.5	0.00386
Cmah	114193.5	210028.8	0.04545
Fxr1	5978.25	45279.63	0.00691
Gpam	105199.3	54561.63	0.04368
Hp	33193.75	4639975	<0.00001
Itih3	4721.225	206829.5	0.00249
A2m	1020405	3238275	0.00212
Ddx3x	224062.5	347047.5	0.02264
Ssr4	376690	675063.8	0.00575
Thrsp	188747.5	36390.89	0.02783
Ces1e	362962.5	37051.5	0.02198
Sord	2466100	1195385	0.00703
Cyp2c29	2433125	681237.5	0.01485
Cyp3a11	1188153	19656.98	0.00154
Cyp3a13	9652.875	263736.3	<0.00001

Gstt1	691607.5	481100	0.0462
A2mp	3488.825	758858.6	0.04701
Sec24d	288970	490816.3	0.02222
Eif4g1	80844.5	133271.3	0.03907
Uggt1	156152.3	277447.5	0.01719
Srsf1	94732.75	173285.3	0.04659
Tha1	0	233985	0.01005
Rps27	666435	1101993	0.01498
Slc39a14	60185.75	461945	0.00025
Ccdc25	29321.75	153385.9	0.04611
Ostc	610742.5	1023999	0.00627
Ldhd	660100	1312050	0.00027
Mogs	212247.5	328292.5	0.03392
Acat3	2311475	1731800	0.04488
Mup21	138477.5	13775	<0.00001
Acsm5	799935	432478.8	0.01099
2810007J24Rik	2311350	1082123	0.00734
Elovl5	106828.8	49887.5	0.02
Ganab	388222.5	591742.5	0.02306
Ces2e	397522.5	159334.4	0.01414
Tmem214	78789.5	224815	0.00057
Dlat	402775	292762.5	0.04808
Lars	88817	135710	0.02342
Qars	125872.8	265758.8	0.01895
Asph	9146.1	105814.5	0.01004
Gmppb	286335	601063.8	0.03703
Dpp9	9333.5	34635.75	0.01863
Qdpr	1164050	861388.8	0.02131
Maob	743600	516886.3	0.04578
Ptges2	5563.975	28001.75	0.01935
Ttpa	265207.5	421458.8	0.02537
Etnppl	104274.3	10880.63	0.00486
Slco2b1	72216.75	34672.38	0.01901
Farsa	79292	170050.1	0.0186
Dnai1	663145	130339.4	0.03711
Cps1	23003250	17080000	0.01732
Mettl7a1	613610	306171.3	0.0055
Emc1	53335.75	135852.1	0.00365
Ufl1	34410.25	76066.38	0.04732
Fam25c	0	88552.13	<0.00001
H6pd	163900	384577.5	0.00111
Eprs	211627.5	322312.5	0.00867
Cml2	201125	312806.3	0.02782
Fermt2	73667.75	279007.5	0.02225
Copa	184580	278037.5	0.03167
Pak2	37971.25	82579	0.01014
Eif3b	127677.5	219867.5	0.04792

Acsl5	622560	412051.3	0.03031
Oplah	195175	357432.5	0.00381
Fgb	236177.5	2213700	<0.00001
Gltpd2	62564.5	244077.9	0.01736
Ugt2b34	762345	512497.5	0.03426
C8a	23208	165944.6	0.00345
Hacd3	96786.75	212500	0.01788
Tat	12668.23	133724.4	0.00513
Ces2a	1003363	603182.5	0.03314
Blmh	317242.5	729345	0.04293
Ugt2b1	1715350	769431.3	0.00393
Ido2	135982.5	81312.63	0.04354
Eppk1	70817.25	182457.5	0.00367
Serpina10	7600.625	234176.3	<0.00001
Dync1li1	24710	46061.5	0.03617
Sds	988577.5	1765763	0.02038
Hpgd	96118.75	1856.838	0.00053
Ces1	548445	239268.8	0.01216
Acaa1b	405732.5	26006.85	0.00734
Fgg	307347.5	2351750	<0.00001
Ces1d	2700550	1272788	0.00138
Akr1d1	686715	439127.5	0.01046
Myh9	664647.5	1004765	0.02869
Hdlbp	478597.5	848096.3	0.01267
Arl8b	41556.5	86816.5	0.01214
Acly	818172.5	365488.8	0.00607
Ddx1	154330	223493.8	0.0338
Ces2c	246717.5	23246.74	0.00113
Gne	188272.5	422862.5	0.00737
Eif3h	90820	141358.8	0.02774
Serpina3n	7566.925	758106.3	<0.00001
Akr1c14	1171038	431945	0.00562
Ces1f	1488800	791796.3	0.02312
As3mt	119076.8	304400	0.01277
Slc3a1	0	85074.5	0.02229
Baat	800112.5	335122.5	0.0006
Hpx	229775	4140613	<0.00001
Cyp2c50	127569.3	21808.25	0.00385
Mat1a	3007650	6508488	0.0115
Yif1a	3470.5	57245.88	0.00603
Dap	88774	292900	0.00646
Ftcd	1735950	1232125	0.02275
Pnpo	165592.5	322487.5	0.00086
Lrg1	0	283562.5	<0.00001
Aldob	14096000	10300550	0.04392
Rpn1	746042.5	1170888	0.00838
Lpgat1	5051.05	207900.1	0.00109

Ugp2	1049050	327742.5	0.00055
Lrp1	166472.5	430482.5	0.00032
Acaa1a	2576150	1726488	0.01495
Tf	944367.5	2062850	0.00131
Pdia5	476957.5	919267.5	0.00215
Mthfd1	872500	616903.8	0.03097
Pcyt2	132240	68693.63	0.00629
MARC2	1848350	1229675	0.00265
Lrrc59	571480	1044766	0.0087
Pdia6	1078385	1799950	0.00567
Steap4	31045.5	944568.8	<0.00001
Ifitm2	10146.75	124754.3	0.02661
Acy1	183247.5	103864.6	0.01525
Gorasp2	158322.5	291960	0.00535
Pls3	268840	440728.8	0.00787
Hagh	550182.5	307187.5	0.02314
Vwa5a	166960	306302.5	0.00129
Clint1	48714	86413.88	0.02671
Lactb2	995065	328432.5	0.00485
Hibadh	587727.5	348862.5	0.02228
Aadac	1427740	794428.8	0.02336
Rrbp1	345035	670905	0.00284
Glo1	5404325	4190913	0.02263
Ndufb5	369037.5	236112.5	0.01309
Kdelr2	39615.25	74924.13	0.02319
Trap1	276595	160957.5	0.01487
Sec61b	216525	444497.5	0.00474
Ddah1	316147.5	176230	0.00674
Manf	106291.8	388498.8	0.00136
Pbld2	392070	236496.3	0.01486
Ndufs4	477452.5	305140	0.0149
Ssr1	205675	593730	0.01102
Spcs2	29342	76815.88	0.04025
Sdhc	1602750	987332.5	0.01274
Shmt2	536887.5	327805	0.01261
Lman1	432210	645423.8	0.04011
Tmed10	257057.5	484946.3	0.0126
Rab1b	15840.75	107742.3	0.02537
Fkbp11	71905	155316	0.03352
Erp44	171170	313626.3	0.00262
Fam114a1	1458.475	28502.13	0.02629
Aacs	306235	44951.25	0.01553
Ephx1	1094528	305650	0.00376
Rpl22l1	31408	76968.13	0.0028
Ppa1	914142.5	1343175	0.01421
Pipox	1187523	650552.5	0.00571
Csad	1176018	450648.8	0.00483

Plin3	105701.3	219821.3	0.00056
Rpn2	561315	923767.5	0.01105
Srpr	50510.5	166684.4	0.00138
Acadsb	345777.5	198792.5	0.03075
Ehhadh	1203125	302881.3	0.00016
Cyp4v2	233700	42736.63	0.00277
Ethe1	459935	288462.5	0.00024
Gstk1	793455	611373.8	0.04808
Cyb5r3	1036793	732593.8	0.03767
Slc38a3	35315.15	437755	0.00056
Keg1	1076320	522068.8	0.00489
Pik3ap1	20204.5	41002.88	0.04141
Vps35	165945	244447.5	0.038
Mvp	96441	291680	0.00017
Hrg	53296.5	127797.1	0.01491
Sdf2l1	30563.75	189022.3	0.00127
Pygl	1617200	848508.8	0.0201
Tm9sf3	90735	208012.1	0.03458
Isyna1	2035.925	483530.1	0.00034
Crybb3	4597.6	354129.3	0.00059
Hyou1	659002.5	1246025	0.00062
Tgm1	22274.5	84415.88	0.03397
Gnmt	6310925	4913325	0.02731
Slco1a1	322892.5	59648.5	0.01074
Copg1	163617.5	291045	0.01654
Hsd17b6	178462.5	26743.53	0.00278
Tbl2	58839.25	155014.4	0.02363
Sec11a	43331.75	125984.5	0.04193
Tmed2	101786.5	178392.5	0.01213
Srsf10	41357.25	106921.9	0.0301
Gda	22926.1	84566	0.00255
Cul1	24377.25	70482.63	0.03415
Hao1	832970	573320	0.00478
Prodh	294970	183597.5	0.02039
Decr2	206377.5	90885.63	0.00975
Bub3	5846.25	51527.38	0.02487
Eif2s3x	304980	427597.5	0.04989
Uso1	79748	124774	0.03136
Sel1l	38099	83782.5	0.01903
Pck1	696667.5	1244180	0.0394

Supplementary table 2: Proteins significantly different in the livers of NC versus C26 mice

Proteins	NC-Mean of LFQ values	C26- Mean of LFQ values	q-value
Rbm8a	13261.86	35080.75	0.03489
Gm10260	1958129	2742400	0.00259
Mgam	10653.71	34433.25	0.03157
Aspg	60646	155802	0.00321
Arfgef2	29766.29	99592.75	0.04145
Slc35d1	63812	130710.4	0.03732
Dhtkd1	612542.9	477135	0.01223
Mup9	6747829	1454736	0.00057
Mup10	2449871	824155	0.00101
Itih4	58241.57	1436120	<0.00001
Mup15	488065.7	24685	0.02027
Slc22a30	116204.9	40677.38	0.02053
Tmed7	175320	298521.3	0.01281
Ces1b	199785.7	66751.75	0.03695
Fga	525747.1	2636750	<0.00001
Apob	154982.9	224691.3	0.00912
Acacb	105215.7	41275.13	0.01503
Mup7	492957.1	51600.91	0.00036
Agl	494224.3	315592.5	0.00596
Aox3	678718.6	223036.3	0.00031
Akr1c19	226737.1	111247.3	0.0306
Sec22b	144374.9	204377.5	0.02866
Kng1	109621.9	414772.5	0.00036
Ngp	985.4571	79978	0.00035
Prdx6	3135886	2583138	0.00718
Dld	1156051	864806.3	0.02295
Prkcsh	299925.7	523840	0.00107
Prdx4	3557114	4325500	0.01925
Faah	173625.7	129114.9	0.03848
Flot1	5886.286	42517.75	0.0016
Atox1	326260	437571.3	0.02153
Gsto1	232470	141831.3	0.03968
Cyp3a25	154146	30464.26	0.00719
Hgd	2349100	1747038	0.00489
Scarb2	144657.1	229531.3	0.00264
Ddt	6835800	5342750	0.01057
Phyh	496185.7	150747.5	0.00337
Slc27a2	933818.6	1244894	0.0105
Bhmt	7625057	10941463	0.00193
Ap1b1	318871.4	379743.8	0.0089
Gstm6	390715.7	172878	0.00195
Calu	121971.7	306761.3	0.00309
Fxn	17403.29	48998.25	0.0286
Gamt	502212.9	311285	0.00289
Ddost	617302.9	903648.8	0.00599
Aox1	46330.86	19511.55	0.02413

B3galt1	4111.529	44647.88	0.00636
Banf1	246971.4	339111.3	0.00324
Copb2	176427.1	244866.3	0.00914
Snca	7839.929	23677.43	0.02871
Acot1	74861.43	24408.5	0.02402
Atp2a2	138085.1	213453.8	0.01511
Nnmt	1905.9	226241.6	0.00056
Eef1b	854400	1128286	0.00594
Nmt1	142371.1	227381.3	0.01336
Ugdh	934605.7	676991.3	0.03971
Wdr1	229232.9	334802.5	0.00288
Papss2	246241.4	125364.3	0.00192
Rdh7	2369600	1754488	0.00123
Dhcr7	117034.6	203197.5	0.00976
Ddc	168087.1	91465.38	0.00319
Creg1	313530	132130	0.00401
Idh1	2042071	1563700	0.01826
Cyp8b1	133383.3	32821.25	0.01563
Lgmn	74117.86	178421.3	0.00257
Cope	327305.7	438466.3	0.01023
Rbm3	70672	156262.8	0.01428
Cyp1a2	1008174	223861.6	0.00093
Adh1	14826000	12542125	0.0275
C3	315257.1	1389813	<0.00001
C4b	249768.6	449170	0.00071
Ighm	93056.57	220749.9	0.0347
Igha	18228.29	68299.63	0.03173
Mt2	0	43656.5	0.00059
Mtnd3	35197.43	107052.3	0.0174
Cfb	116731.7	252357.5	0.00263
Mup3	845035.7	109128.3	0.0006
Got2	3523371	3059275	0.03716
Saa1	4850.044	2039488	<0.00001
Saa2	4509.571	1511625	<0.00001
Ldha	2732629	4157013	0.00032
Gpi	1168529	897228.8	0.00125
Me1	1426171	713102.5	0.00057
Cfh	9307.714	242726.3	<0.00001
Ttr	780930	130229.3	<0.00001
Orm2	20111.43	518508.8	0.00019
Pdia4	738061.4	1499725	0.00031
Hsp90b1	1906057	3198213	0.00109
Mdh2	2860071	2495525	0.03229
P4hb	3628786	5945000	0.00011
Ncl	567602.9	690393.8	0.04517
Apoa2	1330443	966062.5	0.02758
Eef1a1	8595600	10729825	0.00652

Alad	716775.7	561676.3	0.00201
Gstm1	16139571	9295538	0.00027
Fn1	327504.3	937852.5	0.0002
Lamp1	51296.86	211072.9	0.01642
Mup2	377161.4	95253.88	0.00179
Lcn2	11210.29	190927.5	0.01136
Cyp2d9	589394.3	283308.8	0.00071
Otc	5466129	4213300	0.00617
Gas2	57860.71	13667.31	0.02103
Apcs	22493.67	6721688	<0.00001
Fabp1	19523000	12258188	0.00109
Rpl7a	747662.9	1077484	0.00935
Umps	122240	171338.8	0.02435
Scd1	73285.57	3862.125	0.02777
Gpd1	1603771	1300900	0.01746
Rpl27a	1043589	1432525	0.03156
Rpl7	1250443	1790013	0.00065
Mdh1	6205714	5056363	0.00456
Rpsa	1090156	1418388	0.00509
Calr	1606529	2749013	0.00126
Slc2a2	326932.9	67819.1	0.00269
Rplp0	1158271	1517475	0.00239
Hmox1	4152.729	59355.75	0.00124
Gstm2	715827.1	466092.5	0.00381
Ca3	29127286	13971625	<0.00001
Lcat	5540.643	27038.25	0.00886
Pah	1693814	435713.8	0.00034
Mut	470245.7	364636.3	0.04785
Ass1	7759943	5630150	0.00063
Gapdh	10364029	8589138	0.0042
Eno1	3508400	2534413	0.00058
Ptbp1	310965.7	448690	0.02273
Ap2a2	298660	389231.3	0.00448
Selenbp1	631501.4	526983.8	0.03048
Ugt2b17	956892.9	537742.5	0.00126
Hspa1a	96183.57	24702.88	0.00222
Fasn	2791557	986715	0.00036
Gstp1	29194000	17495500	0.00094
Gstm3	382470	215285	0.00044
Cox4i1	1018501	784400	0.0471
Hspa5	4317657	6732875	0.00065
Cyp2a5	119376.1	33702.88	0.00742
Plg	57946.57	139260	0.00079
Tgm2	351760	836512.5	<0.00001
Eif3a	166430	233096.3	0.03974
Ca5a	51915.29	33858.63	0.022
Ces1c	421774.3	180432.5	0.00051

Cat	7257114	2579025	0.00037
Ppib	950988.6	1492225	0.00063
Cyp2d10	1380071	810233.8	0.00038
Rps2	690064.3	900841.3	0.0053
Uox	3182357	1328326	0.00031
Tln1	285548.6	360993.8	0.03714
Hsd3b3	308395.7	109547.6	<0.00001
Ctnna1	173332.9	242155	0.04973
S100a8	12597.63	533161.3	<0.00001
Man2a1	92810.14	254245	<0.00001
Rpl3	668250	962418.8	0.00107
Pdia3	2289514	3403013	0.00071
Cyp17a1	15288.71	150450.3	0.01656
Aco1	1450529	1167588	0.0036
Pabpc1	860771.4	1092923	0.00191
Oat	339298.6	622060	0.01648
Vtn	109719.7	291078.8	0.00038
Gsta3	6308929	3960375	0.0007
Fkbp4	300855.7	186682.5	0.02396
S100a9	46615.8	863938.8	0.00035
Scp2	7082643	4121013	0.00034
Wars	49228.86	131681.8	0.01204
Cyp2f2	1856357	606073.8	0.00075
Apoc3	255101.4	130738	0.00369
Mif	1894357	1435463	0.01094
Ephx2	2180629	1171470	0.0001
Asgr1	763322.9	985302.5	0.01057
Pdha1	400204.3	270336.3	0.03881
Hal	531144.3	399697.5	0.01614
Canx	732727.1	929370	0.04266
Rpl12	962311.4	1215135	0.0389
Rpl18	479141.4	709310	0.00783
Sar1a	50812.14	134621.4	0.01856
Hspa9	1502071	1141713	0.01846
Mbl1	24915.86	114402.3	0.0063
Col18a1	49905.29	106961	0.00123
Vps26a	132511.7	217011.3	0.0146
Inmt	2413571	1138914	0.00037
Rpl28	296614.3	446763.8	0.01026
Acsl1	2505200	1213308	<0.00001
Stat1	139235.4	31815.88	0.00139
Stat3	95840.14	217912.5	0.00061
Cct8	390637.1	301792.5	0.02271
Plin2	105085	289198.8	0.00335
Fkbp2	187314.3	306428.8	0.00646
Fdx1	540304.3	396710	0.01679
Stt3a	263322.9	430795	0.00058

Aldh2	7624229	5017938	0.00057
Aldh3a2	260657.1	92513.75	0.00061
Capza1	91632	128189.4	0.04072
Rpl6	791138.6	1091894	0.00335
Rplp1	3389257	4920963	0.00194
Rpl5	491127.1	654527.5	0.00734
Eif1	314250	512390	0.00106
Cbr1	648947.1	459803.8	0.04166
Tdo2	107280.3	169860	0.00361
Hnrnpa1	94703.86	214418.8	0.00949
Hpd	2342257	1821100	0.03673
Ctsh	431571.4	658502.5	0.03973
Bckdha	1489443	900812.5	0.00409
Ahcy	4966343	4166900	0.02364
Fmo1	493801.4	187592.5	0.00039
Shmt1	2063600	1363613	0.001
Atp6v1a	40973	79077.38	0.01451
Hsd17b4	1078693	784053.8	0.00418
Pon1	907527.1	697740	0.03049
Pkm	169362.6	267590	0.03953
Gck	104992.7	56095.13	0.00525
Rpl10a	862415.7	1238500	0.0007
Dbt	565217.1	397593.8	0.00146
Pklr	1385757	640072.5	0.00029
Idh2	609140	495455	0.02664
St3gal1	8698.143	53057.75	0.00031
Abcd3	471537.1	259483.8	0.0002
Rab8a	14652.86	120773.8	0.00854
Cyb5a	2117457	1655338	0.01204
Erp29	271574.3	415175	0.00337
Actn4	652550	764216.3	0.0385
Tm9sf2	17112.14	45556.75	0.00546
Eef2	1745143	2381825	0.00059
Gulo	735557.1	539240	0.01853
Sec61g	190125.7	346230	0.01404
Eif4a1	542378.6	684733.8	0.01743
Arf3	535390	746005	0.00413
Abce1	116839.4	181201.3	0.04298
Rpl26	378152.9	512303.8	0.03282
Pcbd1	2263386	1744838	0.01787
Sec61a1	109998.7	230547.5	0.00145
Arf4	105684.4	173222.3	0.02225
Abat	1015111	772830	0.003
Copz1	73712.86	118622.3	0.00282
Hnrnpk	609477.1	709391.3	0.0367
Rps15a	462057.1	713622.5	0.01791
Rps14	804055.7	1107235	0.01917

Rps11	322248.6	473307.5	0.00343
Rps13	615395.7	798063.8	0.00595
Rps4x	1381157	1748963	0.03486
Rpl18a	492342.9	644025	0.03802
Rps6	1286639	1960925	0.00802
Rpl23	1209257	1610063	0.02296
Rps26	178845.7	253155	0.03242
Rps28	564484.3	720103.8	0.02294
Rpl31	2882543	4099413	0.00385
Rpl8	687722.9	976147.5	0.00113
Tpt1	369591.4	566890	0.00194
Rps17	1135060	1554888	0.00891
Rps12	616724.3	872391.3	0.00411
Rps10	838520	1194238	0.00107
Phb	856138.6	715415	0.04172
Gnb2l1	943618.6	1295450	0.00411
Tuba4a	664097.1	522620	0.02795
Ebp	1606400	2184238	0.00506
Hnrnph2	256278.6	340861.3	0.02553
Akr1c6	10005629	6118413	0.00281
Gaa	31640	5199.875	0.03006
Cct7	272760	207286.3	0.02696
Cct2	379937.1	313670	0.01021
Cct5	618537.1	516162.5	0.00761
Cct3	430670	326557.5	0.02031
Rpl19	845017.1	1181996	0.03672
Serping1	48930.43	229905	0.00026
Rps3a	1490229	1949450	0.01168
Psme1	357781.4	291483.8	0.04393
G3bp2	76704.71	138174.4	0.01659
Anpep	193510	312065	0.00404
Rps5	326525.7	461401.3	0.0364
Gclc	654737.1	387262.5	0.00061
Cpt1a	522032.9	690406.3	0.04233
Fh	1019880	1184213	0.04584
Ctsc	25615.16	133598	0.00169
Fmo5	1848686	1124823	<0.00001
Tubb5	343030	416225	0.02107
Rplp2	1437786	1873013	0.03122
Prdx5	2015800	1686275	0.01378
Rbp4	84106.86	25867	0.00301
Egfr	405311.4	721083.8	0.0003
Sec23a	346054.3	489170	0.01499
Nme2	1505429	1990925	0.00107
Aqp1	120386.4	184025.9	0.04931
Jup	17073.71	20483.88	0.03201
Nucb1	193814.3	283162.5	0.02888

Atp5a1	4526029	3667313	0.04932
Rac2	14779.71	67018.5	0.00368
Fabp5	416572.9	1403230	<0.00001
Pc	3298386	4220463	0.00716
Clu	59948.29	262145	0.00056
Acads	1279686	1100419	0.02785
Ambp	95934.29	392166.3	0.00231
Gbp8	67668	20667.75	0.01192
Myl12b	319550	445668.8	0.01097
Slc16a10	13212.43	46638.25	0.03717
Pid1	64284.14	175065.8	0.01019
Ugt2b36	5821714	3499000	0.00108
Sdk1	1726757	784262.5	0.00336
Prrc1	76161.86	155094.9	0.00712
Sec31a	368570	527997.5	0.0006
Sult1d1	339937.1	223767.5	0.00978
Slc27a5	840572.9	1004541	0.00492
Cyp2c67	702744.3	184178.8	0.00062
Gls2	489317.1	282250	0.00029
Aldh16a1	83196.43	34697	0.00583
Q502G5	30119.86	58953.38	#N/A
Mup20	103565.6	1910.75	0.01384
Cluh	674097.1	456987.5	0.00663
Acaca	284010	83262.88	0.00943
Dnajc11	25160.71	51028.13	0.03671
Acbd5	208172.9	89920.88	0.00088
Arcn1	255737.1	397363.8	0.00093
A1cf	163352.9	109841.8	0.02842
Orm1	44807.86	1035866	<0.00001
Myl6	569874.3	708117.5	0.04342
Grb2	27845.14	54017.5	0.02164
Flot2	4801.971	56252.75	0.01561
Gcdh	836595.7	652172.5	0.00382
Stip1	229525.7	175186.3	0.00964
Pter	282417.1	402298.8	0.00124
Vdac2	259808.6	200656.3	0.0072
Vdac1	1216543	1012490	0.0125
Adck3	437774.3	74591.63	<0.00001
Gstt2	483445.7	350491.3	0.01656
Cp	76967.71	214351.3	0.0003
Serpinf2	141464.6	345667.5	0.02659
Ctnna2	131584.3	227818.8	0.00602
Cmah	99165.57	210028.8	0.00316
Hp	216408.6	4639975	<0.00001
Hsd3b5	335790	131929.6	0.00605
Itih1	21583.53	105519	0.00688
Itih2	35946.86	86745	0.03884

Itih3	2005.786	206829.5	0.00036
A2m	1175524	3238275	0.00033
Ddx3x	241318.6	347047.5	0.0031
Ssr4	413620	675063.8	0.00026
Sptbn1	116759.7	163220	0.01247
Thrsp	338490	36390.89	0.00037
Ndufa4	1956071	1571213	0.0239
Ugt1a9	1835900	1397850	0.00129
Selenbp2	13727429	8336775	0.00124
Ces3a	4944457	3152088	0.00086
Ugt1a1	840444.3	659403.8	0.0459
Ces1e	337345.7	37051.5	0.00125
Rgn	11165314	9268725	0.03842
Sord	2235271	1195385	0.00062
Cyp2c29	2307071	681237.5	0.00075
Cyp3a11	1038120	19656.98	<0.00001
Cyp3a13	23572.33	263736.3	<0.00001
Gstt1	720550	481100	0.00121
Tpp2	117197.1	75521.25	0.00519
Gpd2	323117.1	251357.5	0.04643
Cltc	1076601	1439425	0.00154
A2mp	3112.129	758858.6	0.00258
Sec24d	282312.9	490816.3	0.00286
Eif4g1	85183.43	133271.3	0.03646
Bckdhb	804994.3	554167.5	0.00335
Snrnp200	13792.43	32818.5	0.04603
Uggt1	165271.4	277447.5	0.0011
Herc4	9682	36164.75	0.04925
Lrpprc	294110	225106.3	0.00678
Tha1	26011.29	233985	0.00414
Cyp2c54	350822.9	117346.1	0.00687
Mlec	112665.6	189837.5	0.01403
Rps9	944254.3	1210600	0.00741
Rps27	739932.9	1101993	0.00975
Fgl1	4635.457	74721.88	0.0031
Slc39a14	55594.43	461945	0.00035
Haao	1778286	1402900	0.00597
Snd1	611815.7	774157.5	0.01464
Ostc	673111.4	1023999	0.00033
Mtch2	476720	369541.3	0.0378
Ndufa12	376328.6	292498.8	0.03164
Tubb2a	742535.7	615993.8	0.00785
Abcg2	82185.29	42038.38	0.02891
Ldhd	734700	1312050	0.00027
Actn1	240887.1	299602.5	0.03967
Mogs	232591.4	328292.5	0.00609
Ddrgk1	15699.57	48268.13	0.00102

Acat3	2217886	1731800	0.01023
Acad11	310058.6	194888.8	0.0025
Bdh1	1411329	1112144	0.01547
Mup21	130719.7	13775	<0.00001
Tufm	1381243	1149199	0.03714
Pef1	22533	77142.25	0.03037
Erlin2	32886.57	82018.5	0.00231
Hnrnpa3	519411.4	605691.3	0.04587
Acsm5	682065.7	432478.8	0.00619
2810007J24Rik	2068000	1082123	0.00058
Gpt2	201577.4	65014.38	0.0256
Trim23	59602.43	125161.5	0.03047
Slc25a12	379135.7	471433.8	0.02662
Gopc	2873.857	20362.75	0.036
Ganab	411698.6	591742.5	0.00382
Ndufv3	82229.29	46262.5	0.04337
Ces2e	427210	159334.4	0.00032
Srsf7	162612.4	217170	0.03981
Tmem214	107040.1	224815	0.00084
Hspa13	31770	129340.5	0.01608
Lars	84085.29	135710	0.0016
Qars	173608.6	265758.8	0.01246
Nars	108182	174890	0.003
Asph	24329.57	105814.5	0.00632
Gmppb	385847.1	601063.8	0.02751
Dpp9	10621.57	34635.75	0.00228
Qdpr	1172864	861388.8	0.00337
Maob	680431.4	516886.3	0.03978
Ttpa	249602.9	421458.8	0.00101
Ugt2a3	1151210	908861.3	0.04995
Acaa2	5964500	4582350	0.00533
Etnppl	77267.43	10880.63	0.00033
Etf1	25803.57	80365.5	0.02893
Fndc3a	50489	80035.13	0.00711
Clptm1l	8979.429	26763.38	0.03948
Slco2b1	70079.86	34672.38	0.00424
Dnai1	512142.9	130339.4	0.01267
Cps1	22364714	17080000	0.00057
Mettl7a1	546618.6	306171.3	0.00061
Acat2	1367730	916893.8	0.00835
Vwa8	101265.4	70546.5	0.02043
Fam25c	0	88552.13	<0.00001
H6pd	176090	384577.5	<0.00001
Eprs	223648.6	322312.5	0.00063
Lonp1	341424.3	212473.8	0.01333
Pdlim5	73098.57	37375.75	0.00714
Fermt2	58337.54	279007.5	0.00193

Copa	184651.4	278037.5	0.00059
Pak2	27658.57	82579	0.00524
Hmgcs1	20422.09	80863.38	0.04641
Acsl5	606851.4	412051.3	0.00424
Slc25a1	517704.3	363002.5	0.00158
Oplah	262357.1	357432.5	0.01672
Fgb	369178.6	2213700	<0.00001
Gltpd2	60754.43	244077.9	0.00288
Ugt2b34	655007.1	512497.5	0.03278
Galm	408295.7	263451.3	0.02167
C8a	72203.43	165944.6	0.00565
Pdxk	215565.7	136089.5	0.0102
Coq9	315137.1	220026.3	0.01016
Sdha	2656271	1997375	0.00518
Hacd3	97084.57	212500	0.0007
Acad10	305470	180698	0.03212
Abca8a	50463	6845.8	0.0148
Afmid	55656.57	5828.625	0.03998
Tat	16288.5	133724.4	0.00106
Ces2a	946588.6	603182.5	0.00108
Gpt	745430	534176.3	0.00314
Ugt2b1	1548014	769431.3	0.0003
Ido2	143802.9	81312.63	0.00159
Eppk1	68804.14	182457.5	0.0003
Serpina10	13715.31	234176.3	<0.00001
Ero1l	11816.29	27653.75	0.03248
Dync1li1	19682.86	46061.5	0.00288
Sdsl	17940.29	72528.5	0.02384
Clybl	172591.4	129291.6	0.02523
Gstm4	61411.14	31975.5	0.04642
Sds	968482.9	1765763	0.00128
Ttc36	418780	235210	0.00369
Dak	2573243	1807338	0.00384
Upb1	386854.3	77780.25	0.00162
Gys2	314781.4	89442.1	0.00545
Hpgd	38794.14	1856.838	0.00132
Ces1	545525.7	239268.8	0.00062
Acaa1b	391198.6	26006.85	0.00044
Dhcr24	63705.86	175263.8	0.0016
Fgg	424035.7	2351750	<0.00001
Ugt2b37	455661.4	173285.6	0.02451
Abhd14b	1488271	1205200	0.04898
Ces1d	2542686	1272788	<0.00001
Akr1d1	658720	439127.5	0.00335
Myh9	651142.9	1004765	0.00033
Hdlbp	542147.1	848096.3	0.00166
Ttc39c	291201.4	96887.75	0.00422

Arl8b	41487.71	86816.5	0.00087
Hnrnpu	275520	358772.5	0.00368
Sec63	61125.57	135105.1	0.03946
Abcc2	60638.29	29013.84	0.03246
Isoc1	395735.7	308455	0.03974
C11orf54	874055.7	647143.8	0.00288
Acly	898637.1	365488.8	0.00038
Ddx1	187302.9	223493.8	0.0177
Gldc	614858.6	400556.3	0.00412
Txndc5	320864.3	408503.8	0.03507
Ces2c	190184.4	23246.74	0.00035
Gne	233432.9	422862.5	0.00106
Eif3h	107603.9	141358.8	0.046
Cecr5	69641.43	18351.75	0.00593
Serpina3n	22926.61	758106.3	<0.00001
Cisd1	649765.7	408418.8	0.04587
Akr1c14	1128184	431945	0.00032
Ces1f	1452114	791796.3	0.00108
As3mt	140896.7	304400	0.00505
Slc3a1	3065.4	85074.5	0.00033
Baat	775585.7	335122.5	<0.00001
Sult5a1	63777.14	6954.25	0.00062
Dcxr	226528.6	137269.6	0.0078
Hpx	315887.1	4140613	<0.00001
Cyp2c50	121008.7	21808.25	0.00064
Mat1a	2878400	6508488	0.00039
Yif1a	11343.14	57245.88	0.00599
Dap	117726.1	292900	0.00038
Ftcd	1717371	1232125	0.00101
Pnpo	228048.6	322487.5	0.01233
Lrg1	7855.257	283562.5	<0.00001
St3gal4	0	56160.4	0.0151
Aldob	14146429	10300550	0.00153
Asl	2417514	1784838	0.00783
Snx4	23257.71	50363.63	0.00884
Dera	122151.6	76413.63	0.00508
Rpn1	788050	1170888	0.00064
Lpgat1	7037.643	207900.1	<0.00001
Grhpr	1440143	1853763	0.01248
Pcca	389001.4	285858.8	0.01828
Ugp2	893140	327742.5	0.00025
Lrp1	189257.1	430482.5	0.00028
Acaa1a	2686029	1726488	0.00277
Tf	1084270	2062850	0.00038
Pdia5	537555.7	919267.5	0.00036
Macrod1	237817.1	131425.8	0.00591
Mthfd1	782470	616903.8	0.01019

Pcyt2	126324.9	68693.63	0.00126
MARC2	1602086	1229675	0.00172
Lrrc59	575951.4	1044766	0.00059
Pdia6	1190816	1799950	0.00099
Steap4	93423.29	944568.8	<0.00001
Sec14l2	1570314	1186341	0.0052
Acy1	162145.7	103864.6	0.00598
Gorasp2	160211.4	291960	0.00035
Eif3m	108698	156678.8	0.01478
Actr3	305810	433466.3	0.04348
Pdxdc1	10027.2	37850.13	0.01678
Pls3	307622.9	440728.8	0.00137
Hagh	507720	307187.5	0.00193
Vwa5a	209890	306302.5	0.00399
Clint1	50527.29	86413.88	0.00359
Lactb2	915080	328432.5	0.00029
Dnajb11	142145.9	354030.4	0.03839
Hibadh	513111.4	348862.5	0.00138
Gstt3	294505.7	193855.6	0.04682
Etfa	3769286	3223925	0.01196
Dpy30	32884	43466.5	0.00563
Mccc1	340215.7	253671.3	0.0433
Pla2g12b	17686.07	46000.63	0.01119
Nudt7	1026709	763605	0.00381
Aadac	1360500	794428.8	0.00094
Rrbp1	378285.7	670905	0.00024
Arhgdia	457117.1	571193.8	0.03671
Ndufa5	265412.9	166788.8	0.01245
Rpl17	669882.9	962997.5	0.00501
Glo1	5011643	4190913	0.00671
Lap3	1968000	1548500	0.00597
Sdhb	973355.7	729401.3	0.00551
Ndufb4	94697	22457.7	0.0024
Sar1b	558744.3	494337.5	0.00931
Ndufb5	327897.1	236112.5	0.00289
Ndufb9	649015.7	532483.8	0.02893
Kdelr2	34298.57	74924.13	0.0005
Trap1	278235.7	160957.5	0.00036
Atp5f1	1136356	911191.3	0.02105
Sec61b	213492.9	444497.5	0.00231
Ndufa6	127582.6	95368.38	0.03657
Ndufab1	513260	312992.5	0.02552
Uqcrfs1	357867.1	287610	0.02455
Ddah1	314730	176230	0.00678
Manf	193686.6	388498.8	0.00712
Pbld2	394424.3	236496.3	0.0017
Rpl11	1175627	1569750	0.0146

Ndufs4	420111.4	305140	0.00426
Ssr1	311527.1	593730	0.02202
Tpd52l2	71132.57	93956.63	0.01917
Uqcrc1	1335600	1122334	0.04186
Sdhc	1446114	987332.5	0.00948
Rpl15	699828.6	1051858	0.00131
Shmt2	506137.1	327805	0.00159
Qrsl1	95916.14	146959.5	0.03011
Rps19	740505.7	920848.8	0.00892
Pdhb	615327.1	404516.3	0.01463
Lman1	471202.9	645423.8	0.01134
Tmed10	286531.4	484946.3	0.00299
Erp44	190385.7	313626.3	0.00264
Fam114a1	6165	28502.13	0.00877
Aacs	335618.6	44951.25	0.00063
Ephx1	943352.9	305650	0.0003
Idh3a	248025.7	167291.3	0.04302
Gbe1	528072.9	332282.5	0.00315
Rpl22l1	38867	76968.13	0.00064
Ppa1	955565.7	1343175	0.00088
Pipox	1109610	650552.5	0.00072
Dnajb4	17352.14	48247.88	0.04957
Ndufa11	52319	23792.38	0.04618
Rpl4	832905.7	1098049	0.00161
Eef1g	581162.9	807750	0.00325
Ociad2	55055.14	29945.13	0.01341
Cnn3	96209	33811.13	0.02418
Uqcrc2	1688600	1354938	0.01656
Dhdh	445764.3	352281.3	0.02222
Csad	1020061	450648.8	0.00032
Cyp27a1	1824557	1203253	0.00628
Plin3	121274.6	219821.3	0.00507
Rpn2	628022.9	923767.5	0.00071
Srpr	86505	166684.4	0.00682
Acot12	100123.7	54228.25	0.02456
Acadsb	372124.3	198792.5	0.00117
Ehhadh	1050211	302881.3	<0.00001
Dmgdh	1605143	1288225	0.00492
Cyp4v2	201965.7	42736.63	0.00032
Pmpca	83092	55456.88	0.02013
Igtp	63559.71	29038.63	0.04957
Ssr3	330018.6	469667.5	0.01223
Cd302	11724.71	65335	0.00679
Pbld1	714327.1	560318.8	0.01852
Paics	225187.1	127782	0.03979
Ethe1	413375.7	288462.5	0.00223
Gstk1	723747.1	611373.8	0.03879

Cyb5r3	1008829	732593.8	0.00137
Slc38a3	54806.34	437755	0.00062
C16orf13	698767.1	429585	0.00881
Ndufb10	670667.1	529450	0.03843
Crip2	272855.7	399730	0.00455
Hoga1	1203334	992618.8	0.00799
Rmdn1	290802.9	232787.5	0.01929
Atp5h	2030943	1799788	0.04537
Iyd	197295.7	261191.3	0.02907
Keg1	1013304	522068.8	0.00029
Mettl7b	658261.4	799931.3	0.03539
Cyp4f14	142342.6	74431.5	0.04458
Upf1	83643.29	121668.8	0.01464
Hsd17b11	120459.1	69039.25	0.00337
Aldh6a1	2394286	1898600	0.00453
Hsd3b7	79622.86	48813.25	0.0397
Dpys	764285.7	603741.3	0.01481
Vps35	198124.3	244447.5	0.04389
Mvp	116588.4	291680	<0.00001
Ndufa13	564414.3	431677.5	0.01016
Sdf2l1	48538.57	189022.3	0.00192
Pygl	1584943	848508.8	0.00137
Tm9sf3	92539	208012.1	0.00533
Cpb2	854.2	51107.21	0.03392
Ivd	608588.6	489196.3	0.01937
Isyna1	386.9843	483530.1	0.00029
Nqo2	670015.7	440302.5	0.0049
Copb1	156351.4	232160	0.00426
Akr1a1	2550529	2141963	0.02893
Ddx21	164115.7	83339	0.01742
Cml1	148729	86107.63	0.0197
Slco1b2	315618.6	238448.8	0.04328
Crybb3	5702.214	354129.3	<0.00001
Gnpnat1	173437.1	235521.3	0.03637
Hyou1	750528.6	1246025	0.00058
Cyp2d22	314595.7	240245	0.02779
Tgm1	25998.86	84415.88	0.00063
Scly	78825.57	108610.3	0.00744
Aldh9a1	1421729	1121515	0.03885
Elovl2	55492.57	24087.63	0.0239
Clca3a1	9966.571	73066.13	0.00224
Acox2	340715.7	271700	0.04423
Gnmt	6090986	4913325	0.00426
Acss2	443270	214686.3	0.00627
Plec	225402.9	446167.5	0.00507
Cnpy2	155949.1	249772.5	0.03984
Slco1a1	296480	59648.5	0.00069

Abcb11	296512.9	191310	0.00598
Slc25a10	830841.4	653546.3	0.01567
Eif3i	173184.7	228703.8	0.04344
Copg1	197424.3	291045	0.00231
Fbxo6	13325.04	81124.25	0.00609
Sgk2	42355	6577.5	0.00497
Hsd17b6	136728	26743.53	0.00063
Tbl2	75170	155014.4	0.00779
Acox1	785580	1002833	0.03696
Dstn	377242.9	430327.5	0.04336
Tmed2	120146.3	178392.5	0.03516
Mpdu1	130937.4	197261.3	0.00385
Srsf10	55034.86	106921.9	0.0471
Gda	38098.36	84566	0.0102
Sqrdl	225110	304463.8	0.02687
Ak2	756617.1	591077.5	0.03197
Hao1	822557.1	573320	0.00033
Prodh	305821.4	183597.5	0.00034
Preb	62643.86	172284.6	0.00429
Eci2	488568.6	409486.3	0.0493
Entpd5	156857.4	62287.75	0.00483
Decr2	224488.6	90885.63	0.00034
Lipa	83142	19117	0.00505
Eif2s3x	318117.1	427597.5	0.00454
Fads2	130001.9	59579.63	0.00125
Pf4	20138.99	78854.25	0.01157
Ddx39b	372408.6	534680	0.00941
Uso1	86310.14	124774	0.00641
Dfna5	393280	233517.5	0.03847
Sel1l	38634.57	83782.5	0.00397
Letm1	211022.9	155928.8	0.013
Sucla2	871305.7	698126.3	0.01588
Pck1	769544.3	1244180	0.00573

Supplementary table 3: Top 100 Pathways identified using significant proteins (p<0.05) and the proteins involved in each pathway when comparing the liver of CT and C26 mice.

Ingenuity Canonical Pathways	-log(p-value)	Z-Score	Proteins
SRP-dependent co-translational protein	47.3	7.07	DDOST,RPL10,RPL10A,RPL12,RPL13,RPL15,RPL17,RPL18,RPL18A,RPL19,RPL22,RPL23,RPL24,RPL26,RPL27,RPL27A,RPL3,RPL31,RPL4,RPL5,RPL7,RPL7A,RPL8,RPLP0,RPN1,RPN2,RPS10,RPS11,RPS12,RPS13,RPS14,RPS17,RPS19,RPS20,RPS24,RPS28,RPS4Y1,RPS5,RPS6,RPSA,SEC11A,SEC61A1,SEC61B,SEC61G,SPCS2,SRP72,SRPRA,SSR1,SSR3,SSR4

targeting to membrane			
Eukaryotic Translation Initiation	41.9	6.93	CP,EIF2S2,EIF2S3,EIF3A,EIF3B,EIF3E,EIF3H,EIF3M,EIF4A1,EIF4G1,PABPC1,RPL10,RPL10A,RPL12,RPL13,RPL15,RPL17,RPL18,RPL18A,RPL19,RPL22,RPL23,RPL24,RPL26,RPL27,RPL27A,RPL3,RPL31,RPL4,RPL5,RPL7,RPL7A,RPL8,RPLP0,RPS10,RPS11,RPS12,RPS13,RPS14,RPS17,RPS19,RPS20,RPS24,RPS28,RPS4Y1,RPS5,RPS6,RPSA
Eukaryotic Translation Elongation	40.9	6.56	EEF1A1,EEF1A2,EEF1B2,EEF1D,EEF1G,EEF2,RPL10,RPL10A,RPL12,RPL13,RPL15,RPL17,RPL18,RPL18A,RPL19,RPL22,RPL23,RPL24,RPL26,RPL27,RPL27A,RPL3,RPL31,RPL4,RPL5,RPL7,RPL7A,RPL8,RPLP0,RPS10,RPS11,RPS12,RPS13,RPS14,RPS17,RPS19,RPS20,RPS24,RPS28,RPS4Y1,RPS5,RPS6,RPSA
EIF2 Signaling	35.4	4.75	EIF2S2,EIF2S3,EIF3A,EIF3B,EIF3E,EIF3H,EIF3M,EIF4A1,EIF4G1,GRB2,HSPA5,PABPC1,PPP1CA,PTBP1,RPL10,RPL10A,RPL12,RPL13,RPL15,RPL17,RPL18,RPL18A,RPL19,RPL22,Rpl22l1,RPL23,RPL24,RPL26,RPL27,RPL27A,RPL3,RPL31,Rpl36,Rpl36a,RPL4,RPL5,RPL7,RPL7A,RPL8,RPLP0,RPS10,RPS11,RPS12,RPS13,RPS14,RPS17,RPS19,RPS20,RPS24,RPS28,RPS4Y1,RPS5,RPS6,RPSA,WARS1
Response of EIF2AK4 (GCN2) to amino acid deficiency	35.1	6.32	EIF2S2,EIF2S3,GCN1,RPL10,RPL10A,RPL12,RPL13,RPL15,RPL17,RPL18,RPL18A,RPL19,RPL22,RPL23,RPL24,RPL26,RPL27,RPL27A,RPL3,RPL31,RPL4,RPL5,RPL7,RPL7A,RPL8,RPLP0,RPS10,RPS11,RPS12,RPS13,RPS14,RPS17,RPS19,RPS20,RPS24,RPS28,RPS4Y1,RPS5,RPS6,RPSA
Selenoamino acid metabolism	33.6	5.22	AHCY,CBS/LOC102724560,CTH,GNMT,INMT,MAT1A,NNMT,PAPSS2,RPL10,RPL10A,RPL12,RPL13,RPL15,RPL17,RPL18,RPL18A,RPL19,RPL22,RPL23,RPL24,RPL26,RPL27,RPL27A,RPL3,RPL31,RPL4,RPL5,RPL7,RPL7A,RPL8,RPLP0,RPS10,RPS11,RPS12,RPS13,RPS14,RPS17,RPS19,RPS20,RPS24,RPS28,RPS4Y1,RPS5,RPS6,RPSA
Nonsense-Mediated Decay (NMD)	32.4	6.32	EIF4G1,MAGOHB,PABPC1,RPL10,RPL10A,RPL12,RPL13,RPL15,RPL17,RPL18,RPL18A,RPL19,RPL22,RPL23,RPL24,RPL26,RPL27,RPL27A,RPL3,RPL31,RPL4,RPL5,RPL7,RPL7A,RPL8,RPLP0,RPS10,RPS11,RPS12,RPS13,RPS14,RPS17,RPS19,RPS20,RPS24,RPS28,RPS4Y1,RPS5,RPS6,RPSA
Eukaryotic Translation Termination	32.1	6.08	RPL10,RPL10A,RPL12,RPL13,RPL15,RPL17,RPL18,RPL18A,RPL19,RPL22,RPL23,RPL24,RPL26,RPL27,RPL27A,RPL3,RPL31,RPL4,RPL5,RPL7,RPL7A,RPL8,RPLP0,RPS10,RPS11,RPS12,RPS13,RPS14,RPS17,RPS19,RPS20,RPS24,RPS28,RPS4Y1,RPS5,RPS6,RPSA

Major pathway of rRNA processing in the nucleolus and cytosol	22.2	6.16	RPL10,RPL10A,RPL12,RPL13,RPL15,RPL17,RPL18,RPL18A,RPL19,RPL22,RPL23,RPL24,RPL26,RPL27,RPL27A,RPL3,RPL31,RPL4,RPL5,RPL7,RPL7A,RPL8,RPLP0,RPS10,RPS11,RPS12,RPS13,RPS14,RPS17,RPS19,RPS20,RPS24,RPS28,RPS4Y1,RPS5,RPS6,RPSA,SNU13
Neutrophil degranulation	20.2	2.14	ACAA1,ACLY,AGL,AHSG,ALAD,ANPEP,AP2A2,C3,CAT,CCT2,COPB1,CREG1,CTSC,DDOST,DERA,DPP7,DYNC1LI1,EEF1A1,EEF2,ERP44,FABP5,FTH1,GPI,GSTP1,HP,HSPA1A/HSPA1B,IDH1,LRG1,MIF,MLEC,MVP,NDUFC2,NME2,OSTF1,PDAP1,PDXK,PIGR,PKM,PLAC8,PNP,PRDX4,PRDX6,PSMD1,PSMD11,PTGES2,PYGL,RAB5C,RAC1,S100A8,S100A9,SERPINA1,SERPINA3,TMT1A,TTR,TUBB,TXNDC5
NRF2-mediated Oxidative Stress Response	16.5	0	ABCC2,AOX1,CAT,CYP1A2,CYP2A6 (includes others),Cyp2c40 (includes others),Cyp2c54 (includes others),CYP2C8,Cyp2d22,Cyp2d9 (includes others),CYP2F1,Cyp3a25 (includes others),CYP3A5,CYP3A7,DNAJB11,DNAJB4,EPHX1,ERP29,FMO1,FTH1,GCLC,Gsta1,GSTA3,GSTK1,GSTM1,Gstm3,GSTM5,Gstm6,GSTP1,HACD3,HMOX1,HSP90B1,NQO2,PPIB,SCARB1,SOD2
LPS/IL-1 Mediated Inhibition of RXR Function	16.1	1.13	ABCC2,ACOX1,ACSL1,ACSL5,ALDH2,ALDH3A2,ALDH6A1,CAT,CRAT,CYP2A6 (includes others),Cyp2c40 (includes others),Cyp2c54 (includes others),CYP2C8,Cyp2d22,Cyp2d9 (includes others),CYP2F1,CYP3A5,CYP3A7,FABP1,FABP5,FMO1,FMO5,Gsta1,GSTA3,GSTK1,GSTM1,Gstm3,GSTM5,Gstm6,GSTP1,HMGCS1,MAOB,PAPSS2,SCARB1,SLC27A5,Sult1d1,XPO1
Peroxisomal protein import	14.5	-2.52	ACAA1,ACOX1,AGXT,BAAT,CAT,CRAT,CROT,DECR2,ECH1,EHHADH,EPHX2,GSTK1,HAO1,HSD17B4,IDH1,NUDT7,PHYH,PIPOX,SCP2
Xenobiotic Metabolism PXR Signaling Pathway	14.1	-4.38	ABCC2,ALDH2,ALDH3A2,ALDH6A1,CAT,CES1,Ces1e,Ces1g,CES3,CYP2C8,CYP3A5,CYP3A7,Gsta1,GSTA3,GSTK1,GSTM1,Gstm3,GSTM5,Gstm6,GSTP1,HSP90B1,MAOB,PPP1CA,SRA1,Sult1d1,UGT1A9 (includes others),UGT2B10,UGT2B17,UGT2B28,UGT2B7
Protein Sorting Signaling Pathway	13.1	5.29	AP1B1,AP1G1,ARCN1,CLINT1,CLTC,COPA,COPB1,COPB2,COPE,COPG1,COPZ1,HMOX1,KDELR2,LMAN1,PREB,RER1,SAR1A,SCARB2,SEC13,SEC23A,SEC23B,SEC24D,SEC31A,TMED10,TMED2,TMED7,TMED9,VPS35
Acute Phase Response Signaling	12.9	3.3	AHSG,AMBP,APCS,C3,C4A/C4B,CFB,CP,F2,FGA,FGB,FGG,FN1,GRB2,HMOX1,HP,HPX,ITIH3,ITIH4,PLG,SAA1,SERPINA1,SERPINA3,SERPINF2,SERPING1,SOD2,STAT3,TF,TTR

Xenobiotic Metabolism Signaling	12.7	0	ABCC2,ALDH2,ALDH3A2,ALDH6A1,CAT,CES1,Ces1e,Ces1g,CES3,CYP1A2,CYP2C8,CYP3A5,CYP3A7,FMO1,FMO5,GCLC,Gsta1,GSTA3,GSTK1,GSTM1,Gstm3,GSTM5,Gstm6,GSTP1,HMOX1,HSP90B1,MAOB,NQO2,SRA1,Sult1d1,UGT1A9 (includes others),UGT2B10,UGT2B17,UGT2B28,UGT2B7
Xenobiotic Metabolism CAR Signaling Pathway	12.5	-3.78	ABCC2,ALDH2,ALDH3A2,ALDH6A1,CYP1A2,CYP2C8,CYP3A5,CYP3A7,EGFR,FMO1,FMO5,Gsta1,GSTA3,GSTK1,GSTM1,Gstm3,GSTM5,Gstm6,GSTP1,HSP90B1,RACK1,SRA1,Sult1d1,UGT1A9 (includes others),UGT2B10,UGT2B17,UGT2B28,UGT2B7
Nicotine Degradation II	11.8	-3.77	AOX1,Aox3,CYP1A2,CYP2A6 (includes others),Cyp2c40 (includes others),CYP2C8,CYP2F1,CYP3A5,CYP3A7,FMO1,FMO5,INMT,UGT1A9 (includes others),UGT2A3,UGT2B10,UGT2B17,UGT2B28,UGT2B7
The citric acid (TCA) cycle and respiratory electron transport	11.6	-3.9	ACO2,DLAT,DLD,FH,GLO1,HAGH,IDH2,IDH3A,IDH3G,L2HGDH,MDH2,ME1,PDHA1,SDHA,SDHB,SDHC,SUCLA2,SUCLG1,VDAC1
Response to elevated platelet cytosolic Ca2+	11.4	4.38	ACTN1,AHSG,CALU,CFL1,CLU,FGA,FGB,FGG,FN1,HSPA5,ITIH3,ITIH4,KNG1,MANF,OLA1,PLG,SERPINA1,SERPINA3,SERPINF2,SERPING1,TF,TUBA4A,WDR1
COPI-mediated anterograde transport	11.2	3.58	ARCN1,ARF3,BET1,COPA,COPB1,COPB2,COPE,COPG1,COPZ1,DYNC1LI1,KDELR2,RAB1B,SPTBN1,TMED10,TMED2,TMED7,TMED9,TUBA4A,TUBB2A,USO1
Nicotine Degradation III	11	-3.36	AOX1,Aox3,CYP1A2,CYP2A6 (includes others),Cyp2c40 (includes others),CYP2C8,CYP2F1,CYP3A5,CYP3A7,FMO5,UGT1A9 (includes others),UGT2A3,UGT2B10,UGT2B17,UGT2B28,UGT2B7
COPII-mediated vesicle transport	10.9	4.12	BET1,CTSC,LMAN1,LMAN2,PREB,RAB1B,SEC13,SEC22B,SEC23A,SEC23B,SEC23IP,SEC24D,SEC31A,SERPINA1,TMED10,TMED2,USO1
TCA Cycle II (Eukaryotic)	10.6	-3.05	ACO1,ACO2,DLD,FH,IDH3A,IDH3G,MDH1,MDH2,SDHA,SDHB,SDHC,SUCLA2,SUCLG1
LXR/RXR Activation	10.5	2.83	ACACA,AHSG,AMBP,APOB,C3,C4A/C4B,CLU,FASN,FGA,HPX,ITIH4,KNG1,PON1,S100A8,SAA1,SCD,SERPINA1,SERPINF2,TF,TTR,VTN

Regulation of eIF4 and p70S6K Signaling	10.4	0	EIF2S2,EIF2S3,EIF3A,EIF3B,EIF3E,EIF3H,EIF3M,EIF4A1,EIF4G1,GRB2,PABPC1,RPS10,RPS11,RPS12,RPS13,RPS14,RPS17,RPS19,RPS20,RPS24,RPS28,RPS4Y1,RPS5,RPS6,RPSA
Xenobiotic Metabolism AHR Signaling Pathway	9.89	-4.12	ABCG2,ALDH2,ALDH3A2,ALDH6A1,CYP1A2,Gsta1,GSTA3,GSTK1,GSTM1,Gstm3,GSTM5,Gstm6,GSTP1,HSP90B1,NQO2,PON1,UGT1A9 (includes others)
Regulation of Insulin-like Growth Factor (IGF) transport and uptake by IGFBPs	9.83	4.47	AHSG,APOB,C3,C4A/C4B,CALU,CP,F2,FGA,FGG,FN1,HSP90B1,KNG1,NUCB1,P4HB,PDIA6,PLG,PRKCSH,SERPINA1,SERPINA10,TF
Post-translational protein phosphorylation	9.26	4.24	AHSG,APOB,C3,C4A/C4B,CALU,CP,FGA,FGG,FN1,HSP90B1,KNG1,NUCB1,P4HB,PDIA6,PRKCSH,SERPINA1,SERPINA10,TF
Cargo concentration in the ER	9.03	3.32	CTSC,LMAN1,LMAN2,PREB,SEC22B,SEC23A,SEC23B,SEC24D,SERPINA1,TMED10,TMED2
mTOR Signaling	8.99	1	EIF3A,EIF3B,EIF3E,EIF3H,EIF3M,EIF4A1,EIF4G1,HMOX1,PRKAB1,RAC1,RAC2,RPS10,RPS11,RPS12,RPS13,RPS14,RPS17,RPS19,RPS20,RPS24,RPS28,RPS4Y1,RPS5,RPS6,RPSA
Coronavirus Replication Pathway	8.75	1.73	ARCN1,COPA,COPB1,COPB2,COPE,COPG1,COPZ1,DDX1,IFITM2,TUBA4A,TUBB,TUBB2A
Aryl Hydrocarbon Receptor Signaling	8.59	0	ALDH2,ALDH3A2,ALDH6A1,CYP1A2,Cyp2c40 (includes others),Cyp2c54 (includes others),CYP2C8,Cyp3a25 (includes others),CYP3A5,CYP3A7,Gsta1,GSTA3,GSTK1,GSTM1,Gstm3,GSTM5,Gstm6,GSTP1,HSP90B1,NQO2,TGM2
Melatonin Degradation I	8.05	-3.21	CYP1A2,CYP2A6 (includes others),Cyp2c40 (includes others),CYP2C8,CYP2F1,CYP3A5,CYP3A7,Sult1d1,UGT1A9 (includes others),UGT2A3,UGT2B10,UGT2B17,UGT2B28,UGT2B7
Mitochondrial Dysfunction	8.04	4.6	ACO2,ATP5MG,ATP5PB,CYB5A,DLAT,DLD,GPX1,Gsta1,GSTK1,GSTM1,GSTP1,Gstt1,IDH2,MAOB,MT-CO2,MT-CO3,NDUFA13,NDUFA6,NDUFA9,NDUFB5,NDUFS4,NDUFS6,PDHA1,PRDX6,PRKAB1,SDHA,SDHB,SDHC,SNCA,SOD2,UQCRC2,VDAC1

Superpathway of Melatonin Degradation	7.88	-3.36	CYP1A2,CYP2A6 (includes others),Cyp2c40 (includes others),CYP2C8,CYP2F1,CYP3A5,CYP3A7,MAOB,Sult1d1,UGT1A9 (includes others),UGT2A3,UGT2B10,UGT2B17,UGT2B28,UGT2B7
DHCR24 Signaling Pathway	7.75	3.58	AHSG,AMBP,APOB,C3,C4A/C4B,CLU,DHCR24,FGA,HMOX1,HPX,ITIH4,KNG1,PON1,SAA1,SCARB1,SERPINA1,SERPINF2,TF,TTR,VTN
PXR/RXR Activation	7.57	-2.5	ABCC2,ALDH3A2,CES3,CYP1A2,CYP2A6 (includes others),CYP2C8,CYP3A5,CYP3A7,G6PC1,GSTM1,PAPSS2,SCD,UGT1A9 (includes others)
Valine Degradation I	7.55	-2.33	ABAT,ACADSB,ALDH6A1,BCKDHB,DBT,DLD,EHHADH,HIBADH,HSD17B4,SDS
FXR/RXR Activation	7.17	-1.09	ABCC2,APOB,BAAT,CYP27A1,CYP8B1,FASN,G6PC1,Gsta1,GSTA3,GSTK1,GSTM1,Gstm3,GSTM5,Gstm6,GSTP1,HMOX1,PKLR,PON1,PRKAB1,SCARB1,SLC27A5
MHC class II antigen presentation	7.16	3.15	AP1B1,AP1G1,AP2A1,AP2A2,AP2M1,CANX,CLTC,CTSC,CTSV,DYNC1LI1,LGMN,SEC13,SEC23A,SEC24D,SEC31A,TUBA4A,TUBB2A
Glutathione-mediated Detoxification	7.14	-2.53	ANPEP,Gsta1,GSTA3,GSTK1,GSTM1,Gstm3,GSTM5,Gstm6,GSTP1,Gstt1,Gstt3
Electron transport, ATP synthesis, and heat production by uncoupling proteins	7.11	-4.24	ATP5MG,ATP5PB,ETFA,LRPPRC,MT-CO2,MT-CO3,NDUFA13,NDUFA6,NDUFA9,NDUFB5,NDUFC2,NDUFS4,NDUFS6,SDHA,SDHB,SDHC,TRAP1,UQCRC2
Phase II - Conjugation of compounds	7.06	-1.7	ABHD14B,ACSM5,AHCY,AS3MT,CYP1A2,MAT1A,NAT1,NNMT,PAPSS2,SLC35D1,UGP2,UGT1A9 (includes others),UGT2A3,UGT2B10,UGT2B17,UGT2B28,UGT2B7
Triacylglycerol Degradation	7.01	-1.9	AADAC,AARS1,ABHD6,ALDH2,CES1,Ces1b/Ces1c,Ces1e,Ces1g,Ces2a/Ces2i,Ces2e,CES3,PRDX6
Coronavirus Pathogenesis Pathway	6.91	-3.13	ATP6V1A,DPP9,EEF1A1,EEF1A2,KNG1,PTGES2,RPS10,RPS11,RPS12,RPS13,RPS14,RPS17,RPS19,RPS20,RPS24,RPS28,RPS4Y1,RPS5,RPS6,RPSA,STAT3
TYSND1 cleaves	6.6	-1.34	ACAA1,ACOX1,HSD17B4,PHYH,SCP2

peroxisomal proteins			
Intra-Golgi and retrograde Golgi-to-ER traffic	6.59	3.71	ARCN1,ARF3,COPA,COPB1,COPB2,COPE,COPG1,COPZ1,DYNC1LI1,KDELR2,MAN1A1,MAN2A1,PLIN3,RAB1B,SEC22B,TMED10,TMED2,TMED7,TMED9,TUBA4A,TUBB2A
Fatty Acid β-oxidation I	6.48	-1.9	ACAA1,Acaa1b,ACAA2,ACSL1,ACSL5,EHHADH,HSD17B4,SCP2,SDS,SLC27A5
XBP1(S) activates chaperone genes	6.39	3.16	DNAJB11,GFPT1,HYOU1,PDIA5,PDIA6,PREB,SEC31A,SRPRA,SSR1,YIF1A
Oxidative Phosphorylation	6.09	-3.87	ATP5MG,ATP5PB,CYB5A,MT-CO2,MT-CO3,NDUFA13,NDUFA6,NDUFA9,NDUFB5,NDUFS4,NDUFS6,SDHA,SDHB,SDHC,UQCRC2
Phase I - Functionalization of compounds	6	-4.02	AADAC,ADH1C,ALDH2,CES1,CES3,CYP1A2,CYP27A1,CYP2A6 (includes others),CYP2C8,CYP2F1,Cyp3a25 (includes others),CYP3A5,CYP3A7,CYP4F12,CYP4V2,CYP8B1,FDX1,MAOB,MTARC2,NQO2
Peroxisomal lipid metabolism	5.74	-1.9	ACBD5,ACOX1,ALDH3A2,CRAT,DECR2,EHHADH,HSD17B4,NUDT7,PHYH,SCP2
Glucose metabolism	5.72	-1.07	ALDOB,G6PC1,GCK,GPI,MDH1,MDH2,PC,PCK1,PKLR,PKM,SEC13,SLC25A10,SLC25A11,SLC25A12
Xenobiotic Metabolism General Signaling Pathway	5.69	-3.5	GCLC,Gsta1,GSTA3,GSTK1,GSTM1,Gstm3,GSTM5,Gstm6,GSTP1,HMOX1,NQO2,UGT1A9 (includes others),UGT2B10, UGT2B17,UGT2B28,UGT2B7
Sirtuin Signaling Pathway	5.68	-0.69	ACLY,APEX1,ATG3,ATP5PB,CPS1,IDH2,LDHD,NDUFA13,NDUFA6,NDUFA9,NDUFB5,NDUFS4,NDUFS6,OTC,PCK1,PDHA1,SDHA,SDHB,SDHC,SLC25A4,SOD2,STAT3,TUBA4A,UQCRC2,VDAC1
Oestrogen Biosynthesis	5.66	-2.33	AKR1C4,CYP1A2,CYP2A6 (includes others),Cyp2c40 (includes others),CYP2C8,CYP2F1,CYP3A5,CYP3A7,HSD17B4,HSD17B6
Coagulation System	5.55	0.71	F2,FGA,FGB,FGG,KNG1,PLG,SERPINA1,SERPINF2
Bupropion Degradation	5.31	-1.89	CYP1A2,CYP2A6 (includes others),Cyp2c40 (includes others),CYP2C8,CYP2F1,CYP3A5,CYP3A7

Caveolar-mediated Endocytosis Signaling	5.26	0	ARCN1,COPA,COPB1,COPB2,COPE,COPG1,COPZ1,EGFR,FLOT1,FLOT2,RAB5C
Class I MHC mediated antigen processing and presentation	5.26	4.04	BLMH,CALR,CANX,CTSV,CUL1,FBXO6,FGA,FGB,FGG,HSPA5,NPEPPS,PDIA3,PSMB10,PSMB5,PSMD1,PSMD11,S100A8,S100A9,SEC13,SEC22B,SEC23A,SEC23B,SEC24D,SEC31A,TPP2,UBE2L3,UFL1
Superpathway of Citrulline Metabolism	5.08	-2.12	ASL,ASS1,CPS1,GLS2,OAT,OTC,PRODH,PRODH2
Oxidative Ethanol Degradation III	4.95	0	ACSL1,ALDH2,ALDH3A2,CYP2A6 (includes others),Cyp2c40 (includes others),Cyp2c54 (includes others),CYP2C8,Cyp2d22,Cyp2d9 (includes others),CYP2F1
Isoleucine Degradation I	4.79	-1.34	ACAA2,ACADSB,ACAT2,DLD,EHHADH,HSD17B4,SDS
Glyoxylate metabolism and glycine degradation	4.77	-3	AGXT,BCKDHB,DBT,DHTKD1,DLAT,DLD,GNMT,HAO1,PDHA1
Insulin Secretion Signaling Pathway	4.77	2.84	DLAT,DLD,EIF2S2,EIF2S3,EIF4A1,EIF4G1,GCK,PABPC1,PC,PDHA1,PDIA3,SEC11A,SEC61A1,SEC61B,SEC61G,SLC2A2,SPCS2,SRP72,SSR1,SSR3,SSR4,STAT3
Serotonin Degradation	4.6	-3.32	ADH1C,ALDH2,ALDH3A2,MAOB,Sult1d1,UGT1A9 (includes others),UGT2A3,UGT2B10,UGT2B17,UGT2B28,UGT2B7
Unfolded protein response	4.6	2.33	CALR,CANX,DNAJB11,DNAJB4,ERO1B,HSP90B1,HSPA1A/HSPA1B,HSPA5,P4HB,PDIA6,SEL1L
Apelin Adipocyte Signaling Pathway	4.55	-2.33	CAT,GPX1,Gsta1,GSTK1,GSTM1,GSTP1,Gstt1,PRDX6,PRKAB1,RAC1,RAC2
Glycine Betaine Degradation	4.53	-0.82	BHMT,PIPOX,SARDH,SDS,SHMT1,SHMT2
Heparan Sulfate	4.51	-2.33	AADAC,AARS1,ALDH2,Ces1b/Ces1c,Ces1e,Ces1g,Ces2a/Ces2i,Ces2e,CES3,PRDX6,Sult1d1

Biosynthesis (Late Stages)			
Glutathione Redox Reactions I	4.51	-2.65	GPX1,Gsta1,GSTK1,GSTM1,GSTP1,Gstt1,PRDX6
Phagosome Maturation	4.29	0	ATP6V1A,CALR,CANX,CTSC,CTSV,Dync1i2,DYNC1LI1,PRDX2,PRDX6,RAB5C,RAC1,RAC2,TUBA4A,TUBB,TUBB2A
Iron homeostasis signalling pathway	4.27		ACO1,ACO2,ATP6V1A,CP,CUL1,EGFR,FTH1,HMOX1,HP,HPX,LRP1,SLC39A14,STAT3,TF
ABC-family proteins mediated transport	4.2	1.73	ABCC2,ABCD3,DERL1,DERL2,EIF2S2,EIF2S3,PEX19,PSMB10,PSMB5,PSMD1,PSMD11,SEL1L
Stearate Biosynthesis I (Animals)	4.09	-1.41	ACSL1,ACSL5,ASPG,Ces1e,DBT,DHCR24,ELOVL2,FASN,LPGAT1,SLC27A5
Heparan Sulphate Biosynthesis	4.05	-2.33	AADAC,AARS1,ALDH2,Ces1b/Ces1c,Ces1e,Ces1g,Ces2a/Ces2i,Ces2e,CES3,PRDX6,Sult1d1
Sulphur amino acid metabolism	3.94	-1	AHCY,BHMT,CBS/LOC102724560,CSAD,CTH,ETHE1,FMO1,MAT1A,SLC25A10
Bile Acid Biosynthesis, Neutral Pathway	3.92	-1.89	Akr1c14,AKR1C4,AKR1D1,BAAT,CYP27A1,CYP8B1,SCP2,SLC27A5
Detoxification of Reactive Oxygen Species	3.87	-0.71	ATOX1,CAT,GPX1,GSTP1,P4HB,PRDX2,PRDX6,SOD2
Regulation of TLR by endogenous ligand	3.86	2.45	APOB,FGA,FGB,FGG,S100A8,S100A9
Iron uptake and transport	3.85	1	ABCG2,ACO1,ALAD,ATP6V1A,CP,CUL1,FTH1,HMOX1,TF
Granzyme A Signaling	3.8	1	APEX1,FN1,LMNB2,NDUFA13,NDUFA6,NDUFA9,NDUFB5,NDUFS4,NDUFS6
Androgen Biosynthesis	3.78	-1	AKR1C4,EBP,Gsta1,HSD17B6,HSD3B2,Hsd3b4 (includes others)

Clathrinid-mediated Endocytosis Signaling	3.76	0	AP1B1,AP1G1,AP2A1,AP2A2,AP2M1,APOB,CLTC,CLU,F2,GRB2,PON1,RAB5C,RAC1,S100A8,SERPINA1,TF
Retinoate Biosynthesis I	3.72	-2.24	ADH1C,Akr1c14,AKR1C4,HSD17B6,RDH11,RDH16,Rdh7
Acetyl-CoA Biosynthesis I (Pyruvate Dehydrogenase Complex)	3.7	-2	DBT,DLAT,DLD,PDHA1
HIF1α Signaling	3.64	1.07	APEX1,GCK,GPI,HMOX1,HSPA1A/HSPA1B,HSPA5,Ldha/RGD1562690,PKM,RAC1,RAC2,RACK1,RPS6,SLC2A2,STAT3,TF,VIM
Gluconeogenesis I	3.6	-2.65	ALDOB,ENO1,GAPDH,GPI,MDH1,MDH2,ME1
Acetone Degradation I (to Methylglyoxal)	3.55	-1.89	CYP1A2,CYP2A6 (includes others),Cyp2c40 (includes others),CYP2C8,CYP2F1,CYP3A5,CYP3A7
Ferroptosis Signaling Pathway	3.44	1.15	ACACA,ARF3,CBS/LOC102724560,CTH,FTH1,G3BP1,GCLC,GLS2,HMOX1,PRKAB1,SLC39A14,STAT3,TF
Mitochondrial protein import	3.43	-0.71	ACO2,IDH3G,LDHD,OTC,PMPCA,SLC25A12,SLC25A4,VDAC1
EPH-Ephrin signalling	3.43	3.16	AP2A1,AP2A2,AP2M1,CFL1,CLTC,MYH9,MYL12A,MYL6,PAK2,RAC1
Gene and protein expression by JAK-STAT signaling after IL-12 stimulation	3.42	-0.82	CFL1,MIF,MTAP,PAK2,RPLP0,SOD2
Remodeling of Epithelial Adherens Junctions	3.38		ACTN1,CTNNA1,CTNNA2,MAPRE1,RAB5C,TUBA4A,TUBB,TUBB2A

Fatty acyl-CoA biosynthesis	3.38	-1.89	ACACA,ACLY,ACSL1,ACSL5,FASN,HACD3,SCD
Insertion of tail-anchored proteins into the endoplasmic reticulum membrane	3.36	0.45	ALDH3A2,CYB5A,HMOX1,SEC61B,SEC61G
Complement System	3.35	-0.45	C3,C4A/C4B,C8A,CFB,CFH,SERPING1
Glutaryl-CoA Degradation	3.2	-2	ACAA2,ACAT2,EHHADH,GCDH,HSD17B4

Supplementary table 4: Top 100 Pathways identified using significant proteins (p<0.05) and the proteins involved in each pathway when comparing the liver of NC and C26 mice.

Ingenuity Canonical Pathways	-log(p-value)	Z-Score	Proteins
SRP-dependent cotranslational protein targeting to membrane	47.6	7.21	DDOST,RPL10A,RPL11,RPL12,RPL14,RPL15,RPL17,RPL18,RPL18A,RPL19,RPL23,RPL26,RPL27A,RPL28,RPL3,RPL31,RPL4,RPL5,RPL6,RPL7,RPL7A,RPL8,RPLP0,RPLP2,RPN1,RPN2,RPS10,RPS11,RPS12,RPS13,RPS14,RPS15A,RPS17,RPS19,RPS2,RPS20,RPS23,RPS26,RPS27A,RPS28,RPS4Y1,RPS5,RPS6,RPS9,RPSA,SEC61A1,SEC61B,SEC61G,SRPRA,SSR1,SSR3,SSR4
Eukaryotic Translation Initiation	44.8	7.21	CP,EIF2S3,EIF3A,EIF3E,EIF3H,EIF3I,EIF3M,EIF4A1,EIF4G1,PABPC1,RPL10A,RPL11,RPL12,RPL14,RPL15,RPL17,RPL18,RPL18A,RPL19,RPL23,RPL26,RPL27A,RPL28,RPL3,RPL31,RPL4,RPL5,RPL6,RPL7,RPL7A,RPL8,RPLP0,RPLP2,RPS10,RPS11,RPS12,RPS13,RPS14,RPS15A,RPS17,RPS19,RPS2,RPS20,RPS23,RPS26,RPS27A,RPS28,RPS4Y1,RPS5,RPS6,RPS9,RPSA
Eukaryotic Translation Elongation	44.6	6.86	EEF1A1,EEF1B2,EEF1D,EEF1G,EEF2,RPL10A,RPL11,RPL12,RPL14,RPL15,RPL17,RPL18,RPL18A,RPL19,RPL23,RPL26,RPL27A,RPL28,RPL3,RPL31,RPL4,RPL5,RPL6,RPL7,RPL7A,RPL8,RPLP0,RPLP2,RPS10,RPS11,RPS12,RPS13,RPS14,RPS15A,RPS17,RPS19,RPS2,RPS20,RPS23,RPS26,RPS27A,RPS28,RPS4Y1,RPS5,RPS6,RPS9,RPSA
Eukaryotic Translation Termination	38.5	6.56	ETF1,RPL10A,RPL11,RPL12,RPL14,RPL15,RPL17,RPL18,RPL18A,RPL19,RPL23,RPL26,RPL27A,RPL28,RPL3,RPL31,RPL4,RPL5,RPL6,RPL7,RPL7A,RPL8,RPLP0,RPLP2,RPS10,RPS11,R

			PS12,RPS13,RPS14,RPS15A,RPS17,RPS19,RPS2,RPS20,RPS23,RPS26,RPS27A,RPS28,RPS4Y1,RPS5,RPS6,RPS9,RPSA
Nonsense-Mediated Decay (NMD)	38.1	6.78	EIF4G1,ETF1,PABPC1,RPL10A,RPL11,RPL12,RPL14,RPL15,RPL17,RPL18,RPL18A,RPL19,RPL23,RPL26,RPL27A,RPL28,RPL3,RPL31,RPL4,RPL5,RPL6,RPL7,RPL7A,RPL8,RPLP0,RPLP2,RPS10,RPS11,RPS12,RPS13,RPS14,RPS15A,RPS17,RPS19,RPS2,RPS20,RPS23,RPS26,RPS27A,RPS28,RPS4Y1,RPS5,RPS6,RPS9,RPSA,UPF1
Selenoamino acid metabolism	37.2	5.94	AHCY,CBS/LOC102724560,GNMT,INMT,MAT1A,NNMT,PAPSS2,RPL10A,RPL11,RPL12,RPL14,RPL15,RPL17,RPL18,RPL18A,RPL19,RPL23,RPL26,RPL27A,RPL28,RPL3,RPL31,RPL4,RPL5,RPL6,RPL7,RPL7A,RPL8,RPLP0,RPLP2,RPS10,RPS11,RPS12,RPS13,RPS14,RPS15A,RPS17,RPS19,RPS2,RPS20,RPS23,RPS26,RPS27A,RPS28,RPS4Y1,RPS5,RPS6,RPS9,RPSA,SCLY
Response of EIF2AK4 (GCN2) to amino acid deficiency	37.1	6.56	EIF2S3,RPL10A,RPL11,RPL12,RPL14,RPL15,RPL17,RPL18,RPL18A,RPL19,RPL23,RPL26,RPL27A,RPL28,RPL3,RPL31,RPL4,RPL5,RPL6,RPL7,RPL7A,RPL8,RPLP0,RPLP2,RPS10,RPS11,RPS12,RPS13,RPS14,RPS15A,RPS17,RPS19,RPS2,RPS20,RPS23,RPS26,RPS27A,RPS28,RPS4Y1,RPS5,RPS6,RPS9,RPSA
EIF2 Signaling	33.7	4.54	EIF2S3,EIF3A,EIF3E,EIF3H,EIF3I,EIF3M,EIF4A1,EIF4G1,GRB2,HSPA5,PABPC1,PTBP1,RPL10A,RPL11,RPL12,RPL14,RPL15,RPL17,RPL18,RPL18A,RPL19,Rpl22l1,RPL23,RPL26,RPL27A,RPL28,RPL3,RPL31,RPL4,RPL5,RPL6,RPL7,RPL7A,RPL8,RPLP0,RPLP2,RPS10,RPS11,RPS12,RPS13,RPS14,RPS15A,RPS17,RPS19,RPS2,RPS20,RPS23,RPS26,RPS27A,RPS28,RPS4Y1,RPS5,RPS6,RPS9,RPSA,WARS1
Major pathway of rRNA processing in the nucleolus and cytosol	26.4	6.33	DDX21,NCL,RPL10A,RPL11,RPL12,RPL14,RPL15,RPL17,RPL18,RPL18A,RPL19,RPL23,RPL26,RPL27A,RPL28,RPL3,RPL31,RPL4,RPL5,RPL6,RPL7,RPL7A,RPL8,RPLP0,RPLP2,RPS10,RPS11,RPS12,RPS13,RPS14,RPS15A,RPS17,RPS19,RPS2,RPS20,RPS23,RPS26,RPS27A,RPS28,RPS4Y1,RPS5,RPS6,RPS9,RPSA
LPS/IL-1 Mediated Inhibition of RXR Function	23.5	1.9	ABCB11,ABCC2,ACOX1,ACOX2,ACSL1,ACSL5,ALDH16A1,ALDH1L1,ALDH2,ALDH3A2,ALDH6A1,ALDH9A1,CAT,CPT1A,CYP2A6 (includes others),Cyp2c40 (includes others),Cyp2c54 (includes others),CYP2C8,Cyp2d22,Cyp2d9 (includes others),CYP2F1,CYP3A5,CYP3A7,FABP1,FABP5,FMO1,FMO5,GSTA3,GSTA5,GSTK1,GSTM1,Gstm3,GSTM4,GSTM5,Gstm6,GSTO1,GSTP1,GSTT2/GSTT2B,HMGCS1,MAOB,PAPSS2,SLC27A2,SLC27A5,SLCO1B3,SULT1B1,Sult1d1,Sult2a8,XPO1
NRF2-mediated Oxidative	23.4	-2	ABCC2,AKR1A1,AKR7A2,AOX1,CAT,CBR1,CCT7,CYP1A2,CYP2A6 (includes others),Cyp2c40 (includes others),Cyp2c54 (includes others),CYP2C8,Cyp2d22,Cyp2d9 (includes

Stress Response			others),CYP2F1,Cyp3a25 (includes others),CYP3A5,CYP3A7,CYP4A22,DNAJB11,DNAJB4,DNAJC11,EPHX1,ERP29,FMO1,GCLC,GCLM,GSTA3,GSTA5,GSTK1,GSTM1,Gstm3,GSTM4,GSTM5,Gstm6,GSTO1,GSTP1,GSTT2/GSTT2B,HACD3,HMOX1,HSP90AB1,HSP90B1,NQO2,PPIB,STIP1,VCP
Oxidative Phosphorylation	21.9	-5.4	ATP5F1A,ATP5F1B,ATP5PB,ATP5PD,COX4I1,COX7A2,CYB5A,MT-CO2,MT-ND3,MT-ND5,NDUFA10,NDUFA11,NDUFA12,NDUFA13,NDUFA4,NDUFA5,NDUFA6,NDUFAB1,NDUFB10,NDUFB4,NDUFB5,NDUFB9,NDUFS4,NDUFS6,NDUFS7,NDUFV3,SDHA,SDHB,SDHC,UQCR10,UQCRC1,UQCRC2,UQCRFS1
Neutrophil degranulation	21.2	1.66	ACAA1,ACLY,AGL,ALAD,ANPEP,AP2A2,C3,CAT,CCT2,CCT8,COPB1,CREG1,CTSC,CTSH,DDOST,DDX3X,DERA,DYNC1LI1,EEF1A1,EEF2,ERP44,FABP5,GAA,GPI,GSTP1,HP,HSP90AB1,HSPA1A/HSPA1B,IDH1,IQGAP2,JUP,LAMP1,LAMP2,LCN2,LRG1,MAN2B1,MGAM,MIF,MLEC,MVP,NME2,OSTF1,PDXK,PGAM1,PKM,PNP,PRDX4,PRDX6,PSMD14,PYGL,RAB18,S100A8,S100A9,SERPINA3,SERPINB6,SLC27A2,TMT1A,TTR,TUBB,TXNDC5,VCP
Electron transport, ATP synthesis, and heat production by uncoupling proteins	21.1	-5.58	ATP5F1A,ATP5F1B,ATP5PB,ATP5PD,COX4I1,ETFA,ETFB,LRPPRC,MT-CO2,MT-ND3,MT-ND5,NDUFA10,NDUFA11,NDUFA12,NDUFA13,NDUFA4,NDUFA5,NDUFA6,NDUFAB1,NDUFB10,NDUFB4,NDUFB5,NDUFB9,NDUFS4,NDUFS6,NDUFS7,NDUFV3,SDHA,SDHB,SDHC,TRAP1,UQCR10,UQCRC1,UQCRC2,UQCRFS1
Xenobiotic Metabolism PXR Signaling Pathway	20.5	-5.19	ABCC2,ALDH16A1,ALDH1L1,ALDH2,ALDH3A2,ALDH6A1,ALDH9A1,CAT,CES1,Ces1e,Ces1g,CES3,CYP2C8,CYP3A5,CYP3A7,GSTA3,GSTA5,GSTK1,GSTM1,Gstm3,GSTM4,GSTM5,Gstm6,GSTO1,GSTP1,GSTT2/GSTT2B,HSP90AB1,HSP90B1,MAOB,SRA1,SULT1B1,Sult1d1,Sult2a8,UGT1A1,UGT1A9 (includes others),UGT2B10,UGT2B17,UGT2B28,UGT2B7
Xenobiotic Metabolism CAR Signaling Pathway	19.7	-4.77	ABCC2,ALDH16A1,ALDH1L1,ALDH2,ALDH3A2,ALDH6A1,ALDH9A1,CYP1A2,CYP2C8,CYP3A5,CYP3A7,EGFR,FMO1,FMO5,GSTA3,GSTA5,GSTK1,GSTM1,Gstm3,GSTM4,GSTM5,Gstm6,GSTO1,GSTP1,GSTT2/GSTT2B,HSP90AB1,HSP90B1,RACK1,SRA1,SULT1B1,Sult1d1,Sult2a8,UGT1A1,UGT1A9 (includes others),UGT2B10,UGT2B17,UGT2B28,UGT2B7
Mitochondrial Dysfunction	18.7	6.02	ACO2,ATP5F1A,ATP5F1B,ATP5PB,ATP5PD,COX4I1,COX7A2,CYB5A,DLD,GPD2,GSTA5,GSTK1,GSTM1,GSTP1,Gstt1,GSTT2/GSTT2B,IDH2,MAOB,MT-CO2,MT-ND3,MT-ND5,NDUFA10,NDUFA11,NDUFA12,NDUFA13,NDUFA4,NDUFA5,NDUFA6,NDUFAB1,NDUFB10,NDUFB4,NDUFB5,NDUFB9,NDUFS4,NDUFS6,NDUFS7,NDUFV3,PDHA1,PDHB,PRD

			X6,SAMM50,SDHA,SDHB,SDHC,SNCA,UQCR10,UQCRC1,UQCRC2,UQCRFS1,VDAC1,VDAC2
Xenobiotic Metabolism Signaling	18.5		ABCC2,ALDH16A1,ALDH1L1,ALDH2,ALDH3A2,ALDH6A1,ALDH9A1,CAT,CES1,Ces1e,Ces1g,CES3,CYP1A2,CYP2C8,CYP3A5,CYP3A7,FMO1,FMO5,GCLC,GSTA3,GSTA5,GSTK1,GSTM1,Gstm3,GSTM4,GSTM5,Gstm6,GSTO1,GSTP1,GSTT2/GSTT2B,HMOX1,HSP90AB1,HSP90B1,MAOB,NQO2,SRA1,SULT1B1,Sult1d1,Sult2a8,UGT1A1,UGT1A9 (includes others),UGT2B10,UGT2B17,UGT2B28,UGT2B7
Xenobiotic Metabolism AHR Signaling Pathway	17.3	-4.6	ABCG2,ALDH16A1,ALDH1L1,ALDH2,ALDH3A2,ALDH6A1,ALDH9A1,CYP1A2,GSTA3,GSTA5,GSTK1,GSTM1,Gstm3,GSTM4,GSTM5,Gstm6,GSTO1,GSTP1,GSTT2/GSTT2B,HSP90AB1,HSP90B1,NQO2,PON1,UGT1A1,UGT1A9 (includes others)
Peroxisomal protein import	17.1	-2.98	ACAA1,ACOX1,ACOX2,AMACR,BAAT,CAT,DECR2,ECH1,ECI2,EHHADH,EPHX2,GSTK1,HAO1,HSD17B4,IDH1,NUDT7,PEX5,PHYH,PIPOX,RPS27A,SCP2,SLC27A2
Sirtuin Signaling Pathway	17	1	ACLY,ACSS2,ATG3,ATP5F1A,ATP5F1B,ATP5PB,CPS1,CPT1A,GOT2,IDH2,LDHA,LDHD,MT-ND3,MT-ND5,NDUFA10,NDUFA11,NDUFA12,NDUFA13,NDUFA4,NDUFA5,NDUFA6,NDUFAB1,NDUFB10,NDUFB4,NDUFB5,NDUFB9,NDUFS4,NDUFS6,NDUFS7,NDUFV3,OTC,PCK1,PDHA1,PGAM1,SDHA,SDHB,SDHC,STAT3,TIMM44,TUBA1B,TUBA4A,UQCRC2,UQCRFS1,VDAC1,VDAC2
Aryl Hydrocarbon Receptor Signaling	13.1		ALDH16A1,ALDH1L1,ALDH2,ALDH3A2,ALDH6A1,ALDH9A1,CYP1A2,Cyp2c40 (includes others),Cyp2c54 (includes others),CYP2C8,Cyp3a25 (includes others),CYP3A5,CYP3A7,GSTA3,GSTA5,GSTK1,GSTM1,Gstm3,GSTM4,GSTM5,Gstm6,GSTO1,GSTP1,GSTT2/GSTT2B,HSP90AB1,HSP90B1,NQO2,TGM2
Nicotine Degradation II	13	-4.02	AOX1,Aox3,CYP1A2,CYP2A6 (includes others),Cyp2c40 (includes others),CYP2C8,CYP2F1,CYP3A5,CYP3A7,FMO1,FMO5,INMT,UGT1A1,UGT1A9 (includes others),UGT2A3,UGT2B10,UGT2B17,UGT2B28,UGT2B7,UGT3A1
Response to elevated platelet cytosolic Ca2+	12.9	4.71	A2M,ACTN1,ACTN4,CALU,CFL1,CLU,FGA,FGB,FGG,FN1,HSPA5,ITIH3,ITIH4,KNG1,LAMP2,MANF,PF4,PLG,SERPINA3,SERPINF2,SERPING1,STXBP3,TF,TLN1,TUBA4A,WDR1
Acute Phase Response Signaling	12.5	3.77	A2M,AMBP,APCS,APOA2,C3,C4A/C4B,CFB,CP,FGA,FGB,FGG,FN1,GRB2,HMOX1,HNRNPK,HP,HPX,ITIH2,ITIH3,ITIH4,PLG,RBP4,SAA1,SERPINA3,SERPINF2,SERPING1,STAT3,TF,TTR

Nicotine Degradation III	12.4	-3.64	AOX1,Aox3,CYP1A2,CYP2A6 (includes others),Cyp2c40 (includes others),CYP2C8,CYP2F1,CYP3A5,CYP3A7,FMO5,UGT1A1,UGT1A9 (includes others),UGT2A3,UGT2B10,UGT2B17,UGT2B28,UGT2B7,UGT3A1
Granzyme A Signaling	12.3	3.44	FN1,MT-ND3,MT-ND5,NDUFA10,NDUFA11,NDUFA12,NDUFA13,NDUFA4,NDUFA5,NDUFA6,NDUFAB1,NDUFB10,NDUFB4,NDUFB5,NDUFB9,NDUFS4,NDUFS6,NDUFS7,NDUFV3
Regulation of eIF4 and p70S6K Signaling	12.3		EIF2S3,EIF3A,EIF3E,EIF3H,EIF3I,EIF3M,EIF4A1,EIF4G1,GRB2,PABPC1,RPS10,RPS11,RPS12,RPS13,RPS14,RPS15A,RPS17,RPS19,RPS2,RPS20,RPS23,RPS26,RPS27A,RPS28,RPS4Y1,RPS5,RPS6,RPS9,RPSA
Protein Sorting Signaling Pathway	11.8	5.29	AP1B1,ARCN1,ARFGEF2,CLINT1,CLTC,COPA,COPB1,COPB2,COPE,COPG1,COPZ1,HMOX1,KDELR2,LMAN1,PREB,SAR1A,SCARB2,SEC13,SEC23A,SEC24C,SEC24D,SEC31A,SNX4,TMED10,TMED2,TMED7,VPS26A,VPS35
COPI-mediated anterograde transport	11.2	3.27	ARCN1,ARF3,ARF4,CAPZA1,COPA,COPB1,COPB2,COPE,COPG1,COPZ1,DYNC1LI1,KDELR2,RAB1A,SPTBN1,TMED10,TMED2,TMED7,TUBA1B,TUBA4A,TUBB2A,USO1
Glutathione-mediated Detoxification	10.9	-3.05	ANPEP,GSTA3,GSTA5,GSTK1,GSTM1,Gstm3,GSTM4,GSTM5,Gstm6,GSTO1,GSTP1,Gstt1,GSTT2/GSTT2B,Gstt3,Nat8f2
The citric acid (TCA) cycle and respiratory electron transport	10.7	-3.44	ACO2,DLD,DLST,FH,GLO1,HAGH,IDH2,IDH3A,L2HGDH,LDHA,MDH2,ME1,PDHA1,PDHB,SDHA,SDHB,SDHC,SUCLA2,VDAC1
LXR/RXR Activation	10.4	2.06	ACACA,AMBP,APOA2,APOB,C3,C4A/C4B,CLU,FASN,FGA,HPX,ITIH4,KNG1,LCAT,PON1,RBP4,S100A8,SAA1,SCD,SERPINF2,TF,TTR,VTN
Melatonin Degradation I	10.2	-3.64	CYP1A2,CYP2A6 (includes others),Cyp2c40 (includes others),CYP2C8,CYP2F1,CYP3A5,CYP3A7,SULT1B1,Sult1d1,UGT1A1,UGT1A9 (includes others),UGT2A3,UGT2B10,UGT2B17,UGT2B28,UGT2B7,UGT3A1
mTOR Signaling	9.95		EIF3A,EIF3E,EIF3H,EIF3I,EIF3M,EIF4A1,EIF4G1,HMOX1,RAC2,RPS10,RPS11,RPS12,RPS13,RPS14,RPS15A,RPS17,RPS19,RPS2,RPS20,RPS23,RPS26,RPS27A,RPS28,RPS4Y1,RPS5,RPS6,RPS9,RPSA

PXR/RXR Activation	9.84	-3.5	ABCB11,ABCC2,ALDH3A2,CES3,CPT1A,CYP1A2,CYP2A6 (includes others),CYP2C8,CYP3A5,CYP3A7,GSTM1,PAPSS2,SCD,SLCO1B3,UGT1A1,UGT1A9 (includes others)
Superpathway of Melatonin Degradation	9.84	-3.77	CYP1A2,CYP2A6 (includes others),Cyp2c40 (includes others),CYP2C8,CYP2F1,CYP3A5,CYP3A7,MAOB,SULT1B1,Sult1d1,UGT1A1,UGT1A9 (includes others),UGT2A3,UGT2B10,UGT2B17,UGT2B28,UGT2B7,UGT3A1
Valine Degradation I	9.38	-2.71	ABAT,ACAD8,ACADSB,ALDH6A1,BCKDHA,BCKDHB,DBT,DLD,EHHADH,HIBADH,HSD17B4,SDS
Coronavirus Replication Pathway	9.27	1.39	ARCN1,COPA,COPB1,COPB2,COPE,COPG1,COPZ1,DDX1,IFITM2,TUBA1B,TUBA4A,TUBB,TUBB2A
Fatty Acid β-oxidation I	9.14	-1.94	ACAA1,Acaa1b,ACAA2,ACSL1,ACSL5,ECI2,EHHADH,HSD17B4,IVD,SCP2,SDS,SLC27A2,SLC27A5
Glyoxylate metabolism and glycine degradation	9.04	-3.21	BCKDHA,BCKDHB,DBT,DHTKD1,DLD,DLST,GNMT,GOT2,GRHPR,HAO1,HOGA1,NDUFAB1,PDHA1,PDHB
Neutrophil Extracellular Trap Signaling Pathway	8.94	-3.57	ATP5F1A,ATP5F1B,ATP5PB,COL18A1,Igha,IGHM,LCAT,MT-ND3,MT-ND5,NDUFA10,NDUFA11,NDUFA12,NDUFA13,NDUFA4,NDUFA5,NDUFA6,NDUFAB1,NDUFB10,NDUFB4,NDUFB5,NDUFB9,NDUFS4,NDUFS6,NDUFS7,NDUFV3,PDIA3,PF4,PLA2G12B,PRDX6,RAC2,SDHA,SDHB,SDHC,TIMM44,UQCRC2,UQCRFS1,VDAC1,VDAC2
TCA Cycle II (Eukaryotic)	8.79	-2.89	ACO1,ACO2,DLD,DLST,FH,IDH3A,MDH1,MDH2,SDHA,SDHB,SDHC,SUCLA2
Coronavirus Pathogenesis Pathway	8.61	-3.4	ATP6V1A,DPP9,EEF1A1,KNG1,RPS10,RPS11,RPS12,RPS13,RPS14,RPS15A,RPS17,RPS19,RPS2,RPS20,RPS23,RPS26,RPS27A,RPS28,RPS4Y1,RPS5,RPS6,RPS9,RPSA,STAT1,STAT3
Phase II - Conjugation of compounds	8.56	-2.68	ABHD14B,ACSM5,AHCY,AS3MT,CYP1A2,MAT1A,NNMT,PAPSS2,SLC35D1,SULT1B1,UGDH,UGP2,UGT1A1,UGT1A9 (includes others),UGT2A3,UGT2B10,UGT2B17,UGT2B28,UGT2B7,UGT3A1
Post-translational protein phosphorylation	8.43	3.77	APOA2,APOB,C3,C4A/C4B,CALU,CP,FGA,FGG,FN1,HSP90B1,ITIH2,KNG1,NUCB1,P4HB,PDIA6,PRKCSH,SERPINA10,TF

Estrogen Receptor Signaling	8.4	0	ATP5F1A,ATP5F1B,ATP5PB,CFL1,DDX5,EGFR,GNAI3,GRB2,HSP90AB1,HSP90B1,MT-ND3,MT-ND5,MYL12A,MYL6,NDUFA10,NDUFA11,NDUFA12,NDUFA13,NDUFA4,NDUFA5,NDUFA6,NDUFAB1,NDUFB10,NDUFB4,NDUFB5,NDUFB9,NDUFS4,NDUFS6,NDUFS7,NDUFV3,PDIA3,SDHA,SDHB,SDHC,SRA1,UQCRC2,UQCRFS1
DHCR24 Signaling Pathway	8.29	2.98	AMBP,APOA2,APOB,C3,C4A/C4B,CLU,CYP17A1,DHCR24,DHCR7,FGA,HMOX1,HPX,ITIH4,KNG1,LCAT,PON1,RBP4,SAA1,SERPINF2,TF,TTR,VTN
COPII-mediated vesicle transport	8.14	3.36	CTSC,LMAN1,PREB,RAB1A,SAR1B,SEC13,SEC22B,SEC23A,SEC23IP,SEC24C,SEC24D,SEC31A,TMED10,TMED2,USO1
Regulation of Insulin-like Growth Factor (IGF) transport and uptake by IGFBPs	8.13	3.9	APOA2,APOB,C3,C4A/C4B,CALU,CP,FGA,FGG,FN1,HSP90B1,ITIH2,KNG1,NUCB1,P4HB,PDIA6,PLG,PRKCSH,SERPINA10,TF
Serotonin Degradation	8.04	-4	ADH1C,AKR1A1,ALDH2,ALDH3A2,ALDH9A1,MAOB,SULT1B1,Sult1d1,UGT1A1,UGT1A9 (includes others),UGT2A3,UGT2B10,UGT2B17,UGT2B28,UGT2B7,UGT3A1
MHC class II antigen presentation	7.9	2.52	AP1B1,AP2A2,CANX,CAPZA1,CLTC,CTSC,CTSH,CTSV,DYNC1LI1,LGMN,SAR1B,SEC13,SEC23A,SEC24C,SEC24D,SEC31A,TUBA1B,TUBA4A,TUBB2A
Xenobiotic Metabolism General Signaling Pathway	7.74	-4.02	GCLC,GSTA3,GSTA5,GSTK1,GSTM1,Gstm3,GSTM4,GSTM5,Gstm6,GSTO1,GSTP1,GSTT2/GSTT2B,HMOX1,NQO2,UGT1A1,UGT1A9 (includes others),UGT2B10,UGT2B17,UGT2B28,UGT2B7
Phase I - Functionalization of compounds	7.61	-4.08	AADAC,ACSS2,ADH1C,ALDH2,CES1,CES3,CYP17A1,CYP1A2,CYP27A1,CYP2A6 (includes others),CYP2C8,CYP2F1,Cyp3a25 (includes others),CYP3A5,CYP3A7,CYP4A22,CYP4F12,CYP4V2,CYP7B1,CYP8B1,FDX1,MAOB,MTARC2,NQO2
FXR/RXR Activation	7.58	-2.71	ABCB11,ABCC2,APOB,BAAT,CYP27A1,CYP8B1,FASN,GCLM,GSTA3,GSTA5,GSTK1,GSTM1,Gstm3,GSTM4,GSTM5,Gstm6,GSTO1,GSTP1,GSTT2/GSTT2B,HMOX1,PKLR,PON1,SLC27A5
Glucocorticoid Receptor Signaling	7.45		A2M,ATP5F1A,ATP5F1B,ATP5PB,EGFR,FGG,FKBP4,GRB2,HP,HSP90AB1,HSP90B1,HSPA1A/HSPA1B,HSPA5,HSPA9,MT-ND3,MT-ND5,NDUFA10,NDUFA11,NDUFA12,NDUFA13,NDUFA4,NDUFA5,NDUFA6,NDUFAB1,NDUFB10,NDUFB4,NDUFB5,NDU

			FB9,NDUFS4,NDUFS6,NDUFS7,NDUFV3,PC,PCK1,PLA2G12B,SDHA,SDHB,SDHC,SRA1,STAT1,STAT3,TAT,UQCRC2,UQCRFS1
Cargo concentration in the ER	7.31	2.53	CTSC,LMAN1,PREB,SAR1B,SEC22B,SEC23A,SEC24C,SEC24D,TMED10,TMED2
Peroxisomal lipid metabolism	7.12	-2.31	ACBD5,ACOX1,ACOX2,ALDH3A2,AMACR,DECR2,EHHADH,HSD17B4,NUDT7,PHYH,SCP2,SLC27A2
Intra-Golgi and retrograde Golgi-to-ER traffic	6.94	3.54	ARCN1,ARF3,ARF4,CAPZA1,COPA,COPB1,COPB2,COPE,COPG1,COPZ1,DYNC1LI1,KDELR2,MAN2A1,PLIN3,RAB18,RAB1A,SEC22B,TMED10,TMED2,TMED7,TUBA1B,TUBA4A,TUBB2A
XBP1(S) activates chaperone genes	6.9	3.32	DNAJB11,GFPT1,HYOU1,PDIA5,PDIA6,PREB,SEC31A,SRPRA,SSR1,TLN1,YIF1A
Iron homeostasis signalling pathway	6.84	0	ACO1,ACO2,ATP6V1A,ATP6V1E1,CP,CUL1,EGFR,FXN,HMOX1,HP,HPX,HSPA9,ISCU,LRP1,SKP1,SLC39A14,STAT3,TF,TFR2
Iron uptake and transport	6.59	1.94	ABCG2,ACO1,ALAD,ATP6V1A,ATP6V1E1,CP,CUL1,HMOX1,LCN2,RPS27A,SKP1,TF,TFR2
Triacylglycerol Degradation	6.45	-3	AADAC,ALDH2,CES1,Ces1b/Ces1c,Ces1e,Ces1f,Ces1g,Ces2a,Ces2e,CES3,FAAH,PRDX6
TYSND1 cleaves peroxisomal proteins	6.33	-1.34	ACAA1,ACOX1,HSD17B4,PHYH,SCP2
Mitochondrial protein import	6.22	-1.73	ACO2,ATP5F1A,ATP5F1B,FXN,HSPA9,LDHD,OTC,PMPCA,SAMM50,SLC25A12,TIMM44,VDAC1
Oxidative Ethanol Degradation III	6.15	-2.24	ACSL1,ACSS2,ALDH2,ALDH3A2,ALDH9A1,CYP2A6 (includes others),Cyp2c40 (includes others),Cyp2c54 (includes others),CYP2C8,Cyp2d22,Cyp2d9 (includes others),CYP2F1
Estrogen Biosynthesis	6.09	-2.53	AKR1C4,CYP1A2,CYP2A6 (includes others),Cyp2c40 (includes others),CYP2C8,CYP2F1,CYP3A5,CYP3A7,HSD17B11,HSD17B4,HSD17B6

Phagosome Maturation	6.04		ATP6V1A,ATP6V1E1,CALR,CANX,CTSC,CTSH,CTSV,DYNC1LI1,LAMP1,LAMP2,PRDX2,PRDX5,PRDX6,RAB5A,RAC2,TUBA1B,TUBA4A,TUBB,TUBB2A
Unfolded protein response	5.58	1.67	CALR,CANX,DNAJB11,DNAJB4,DNAJC11,HSP90B1,HSPA1A/HSPA1B,HSPA5,HSPA9,P4HB,PDIA6,SEL1L,VCP
RHO GTPase cycle	5.5	0.52	ABCD3,ACBD5,ACTN1,ALDH3A2,ARHGDIA,CCT2,CCT6A,CCT7,CLTC,DBT,DDRGK1,DDX39B,FERMT2,FLOT1,FLOT2,GOPC,GRB2,HSP90AB1,IQGAP2,JUP,LETM1,LMAN1,NCKAP1,NDUFA5,PAK2,PICALM,RAC2,SAMM50,SHMT2,SPTBN1,STIP1,TUBA1B,VCP
Androgen Biosynthesis	5.48	-0.82	AKR1C4,CYP17A1,EBP,GSTA5,HSD17B6,HSD3B1,Hsd3b4 (includes others),HSD3B7
Isoleucine Degradation I	5.48	-1.63	ACAA2,ACAD8,ACADSB,ACAT2,DLD,EHHADH,HSD17B4,SDS
Heparan Sulfate Biosynthesis (Late Stages)	5.47	-3	AADAC,ALDH2,Ces1b/Ces1c,Ces1e,Ces1f,Ces1g,Ces2a,Ces2e,CES3,PRDX6,SULT1B1,Sult1d1,Sult2a8
Bile acid and bile salt metabolism	5.43	-2.31	ABCB11,ACOX2,AKR1C4,AKR1D1,AMACR,CYP27A1,CYP7B1,CYP8B1,HSD17B4,HSD3B7,SLC27A2,SLC27A5
Glycine Betaine Degradation	5.35	-0.38	BHMT,DMGDH,PIPOX,SDS,SDSL,SHMT1,SHMT2
Remodeling of Epithelial Adherens Junctions	5.3	0	ACTN1,ACTN4,ACTR3,ARPC1B,CTNNA1,CTNNA2,RAB5A,TUBA1B,TUBA4A,TUBB,TUBB2A
Bile Acid Biosynthesis, Neutral Pathway	5.2	-2.33	Akr1c14,AKR1C4,AKR1D1,AMACR,BAAT,CYP27A1,CYP8B1,HSD3B7,SCP2,SLC27A5
Stearate Biosynthesis I (Animals)	5.09	-1.26	ACSL1,ACSL5,ASPG,Ces1e,CYP4A22,DBT,DHCR24,ELOVL2,FASN,LPGAT1,SLC27A2,SLC27A5
Tryptophan Degradation III (Eukaryotic)	4.98	-1.41	ACAA2,ACAT2,AFMID,EHHADH,GCDH,HAAO,HSD17B4,KMO,TDO2
Metabolism of water-soluble	4.98	-2.67	ACACA,ACACB,AOX1,CYB5A,FASN,GSTO1,MCCC1,MCCC2,MMUT,PC,PCCA,PCCB,PDXK,PNPO

vitamins and cofactors			
Bupropion Degradation	4.97	-1.89	CYP1A2,CYP2A6 (includes others),Cyp2c40 (includes others),CYP2C8,CYP2F1,CYP3A5,CYP3A7
Heparan Sulphate Biosynthesis	4.92	-3	AADAC,ALDH2,Ces1b/Ces1c,Ces1e,Ces1f,Ces1g,Ces2a,Ces2e,CES3,PRDX6,SULT1B1,Sult1d1,Sult2a8
Thyroid Hormone Metabolism II (via Conjugation and/or Degradation)	4.8	-3.16	SULT1B1,Sult1d1,UGT1A1,UGT1A9 (includes others),UGT2A3,UGT2B10,UGT2B17,UGT2B28,UGT2B7,UGT3A1
Caveolar-mediated Endocytosis Signaling	4.77		ARCN1,COPA,COPB1,COPB2,COPE,COPG1,COPZ1,EGFR,FLOT1,FLOT2,RAB5A
Plasma lipoprotein assembly, remodeling, and clearance	4.74	1.15	A2M,AP2A2,APOA2,APOB,CES3,CLTC,HDLBP,LCAT,LIPA,P4HB,RPS27A,SAR1B
Cristae formation	4.54	-1.89	ATP5F1A,ATP5F1B,ATP5PB,ATP5PD,DNAJC11,HSPA9,SAMM50
Regulation of TLR by endogenous ligand	4.54	1.89	APOB,FGA,FGB,FGG,GSDME,S100A8,S100A9
Glucose metabolism	4.45	-0.83	ALDOB,GCK,GOT2,GPI,MDH1,MDH2,PC,PCK1,PKLR,PKM,SEC13,SLC25A10,SLC25A12
Chaperone Mediated Autophagy	4.35	1.63	EEF1A1,HSP90AB1,LAMP2,PLIN2,PLIN3,RPS27A
Glucocorticoid Biosynthesis	4.35	-0.45	CYP17A1,EBP,GSTA5,HSD3B1,Hsd3b4 (includes others),HSD3B7
Sulphur amino acid metabolism	4.28	-0.63	AHCY,BHMT,CBS/LOC102724560,CSAD,ETHE1,FMO1,GOT2,MAT1A,SLC25A10,SQOR

Protein folding	4.28	-1.73	ARFGEF2,CCT2,CCT3,CCT5,CCT6A,CCT7,CCT8,FBXO6,STAT3,TUBA1B,TUBA4A,TUBB2A
Coagulation System	4.18	0.38	A2M,FGA,FGB,FGG,KNG1,PLG,SERPINF2
Glutathione Redox Reactions I	4.18	-2.45	GSTA5,GSTK1,GSTM1,GSTP1,Gstt1,GSTT2/GSTT2B,PRDX6
Gluconeogenesis I	4.1	-2.83	ALDOB,ENO1,GAPDH,GPI,MDH1,MDH2,ME1,PGAM1
Branched-chain α-keto acid Dehydrogenase Complex	4.06	-2	BCKDHA,BCKDHB,DBT,DLD
Acetone Degradation I (to Methylglyoxal)	4.04	-2.12	CYP1A2,CYP2A6 (includes others),Cyp2c40 (includes others),CYP2C8,CYP2F1,CYP3A5,CYP3A7,CYP4A22
Gene and protein expression by JAK-STAT signaling after IL-12 stimulation	4.02	-0.38	CAPZA1,CFL1,GSTO1,HSPA9,MIF,PAK2,RPLP0
γ-linolenate Biosynthesis II (Animals)	4.02	-0.82	ACSL1,ACSL5,CYB5A,FADS2,SLC27A2,SLC27A5
Glycogen metabolism	3.94	-1.89	AGL,GAA,GBE1,GYS2,PYGL,RPS27A,UGP2

Supplementary table 5: Top 100 Pathways identified using significant proteins (p<0.05) and the proteins involved in each pathway when comparing the gastrocnemius of CT and C26 mice

Ingenuity Canonical Pathways	-log(p-value)	z-score	Proteins
Acute Phase Response Signaling	15.8	2.53	ALB,APOA2,C3,FGA,FGB,FGG,HP,HPX,ITIH3,ITIH4,SAA1,SERPINA1,SERPINA3,SERPINF1,TTR
LXR/RXR Activation	13.5	-0.3	ALB,APOA2,APOA4,C3,ECHS1,FGA,HPX,ITIH4,SAA1,SERPINA1,SERPINF1,TTR

DHCR24 Signaling Pathway	10.8	-0.3	ALB,APOA2,APOA4,C3,FGA,HPX,ITIH4,SAA1,SERPINA1,SERPINF1,TTR
Striated Muscle Contraction	10.1	1.13	DES,MYL3,TCAP,TMOD1,TNNC1,TNNI1,TNNT1
Response to elevated platelet cytosolic Ca2+	7.39	1.41	ALB,FGA,FGB,FGG,ITIH3,ITIH4,SERPINA1,SERPINA3
Post-translational protein phosphorylation	6.94	-0.38	ALB,APOA2,C3,FGA,FGG,LGALS1,SERPINA1
Regulation of Insulin-like Growth Factor (IGF) transport and uptake by IGFBPs	6.48	-0.38	ALB,APOA2,C3,FGA,FGG,LGALS1,SERPINA1
Cachexia Signaling Pathway	6.04	2.53	ALB,CAPN3,CAPN5,CAPNS1,FGA,FGB,FGG,PSMA6,PSMC1,SERPINA1
Dilated Cardiomyopathy Signaling Pathway	5.96	-2.65	DES,MYH2,MYH7,MYL3,TNNC1,TNNI1,TNNT1
Calcium Signaling	5.84		ATP2A1,MYH2,MYH7,MYL3,PPP3CB,TNNC1,TNNI1,TNNT1
SRP-dependent cotranslational protein targeting to membrane	5.49	-2.45	RPL10A,RPN1,RPS14,RPS17,RPS19,RPS4Y1
Nonsense-Mediated Decay (NMD)	5.38	-1.63	PPP2R1A,RPL10A,RPS14,RPS17,RPS19,RPS4Y1
Coagulation System	5.17	2	FGA,FGB,FGG,SERPINA1
Senescence Pathway	4.9	2.12	CAPN3,CAPN5,CAPNS1,CAT,PDK4,PPP2R1A,PPP3CB,SAA1
Intrinsic Prothrombin Activation Pathway	4.8	1	COL1A2,FGA,FGB,FGG

Selenoamino acid metabolism	4.76	-1.63	RPL10A,RPS14,RPS17,RPS19,RPS4Y1,TXNRD1
Eukaryotic Translation Elongation	4.6	-2.24	RPL10A,RPS14,RPS17,RPS19,RPS4Y1
Eukaryotic Translation Termination	4.58	-2.24	RPL10A,RPS14,RPS17,RPS19,RPS4Y1
Extrinsic Prothrombin Activation Pathway	4.48		FGA,FGB,FGG
nNOS Signaling in Neurons	4.47		CAPN3,CAPN5,CAPNS1,PPP3CB
Response of EIF2AK4 (GCN2) to amino acid deficiency	4.46	-2.24	RPL10A,RPS14,RPS17,RPS19,RPS4Y1
Neutrophil degranulation	4.34	1	C3,CAT,CTSD,HP,NDUFC2,RAB10,SERPINA1,SERPINA3,TTR
Production of Nitric Oxide and Reactive Oxygen Species in Macrophages	4.13	-2.45	ALB,APOA2,APOA4,CAT,PPP2R1A,SERPINA1
Huntington's Disease Signaling	4.11		CAPN3,CAPN5,CAPNS1,CTSD,PSMA6,PSMC1,TGM2
Eukaryotic Translation Initiation	4.06	-2.24	RPL10A,RPS14,RPS17,RPS19,RPS4Y1
Atherosclerosis Signaling	3.95		ALB,APOA2,APOA4,COL1A2,SERPINA1
Binding and Uptake of Ligands by Scavenger Receptors	3.95	0.45	ALB,COL1A2,HP,HPX,SAA1
Regulation of TLR by endogenous ligand	3.76		FGA,FGB,FGG

Amyloid fiber formation	3.76	0	APOA4,FGA,SAA1,TTR
Integrin cell surface interactions	3.62	1	COL1A2,FGA,FGB,FGG
IL-12 Signaling and Production in Macrophages	3.6	0.82	ALB,APOA2,APOA4,C3,COL1A2,SERPINA1
Integrin signaling	3.49		FGA,FGB,FGG
Mitochondrial Dysfunction	3.42	0.38	CAPN3,CAPN5,CAPNS1,CYC1,NDUFB11,PPP3CB,UQCRFS1
RAF/MAP kinase cascade	3.37	2.45	FGA,FGB,FGG,PPP2R1A,PSMA6,PSMC1
Class I MHC mediated antigen processing and presentation	3.36	1.89	CANX,CUL5,FGA,FGB,FGG,PSMA6,PSMC1
Apoptosis Signaling	3.3		CAPN3,CAPN5,CAPNS1,CYC1
Formation of Fibrin Clot (Clotting Cascade)	3.3		FGA,FGB,FGG
Major pathway of rRNA processing in the nucleolus and cytosol	3.29	-2.24	RPL10A,RPS14,RPS17,RPS19,RPS4Y1
Regulation of eIF4 and p70S6K Signaling	3.26		PPP2R1A,RPS14,RPS17,RPS19,RPS4Y1
MyD88:MAL (TIRAP) cascade initiated on plasma membrane	3.25		FGA,FGB,FGG
ILK Signaling	3.11	0.45	MYH2,MYH7,MYL3,NACA,PPP2R1A

Amyloid Processing	3.09		CAPN3,CAPN5,CAPNS1
Clathrin-mediated Endocytosis Signaling	3.04		ALB,APOA2,APOA4,PPP3CB,SERPINA1
Amyotrophic Lateral Sclerosis Signaling	2.98	1	CAPN3,CAPN5,CAPNS1,CAT
mTOR Signaling	2.97		PPP2R1A,RPS14,RPS17,RPS19,RPS4Y1
GP6 Signaling Pathway	2.89	1	COL1A2,FGA,FGB,FGG
TR/RXR Activation	2.86	0	ATP2A1,FGA,HP,MYH7
EIF2 Signaling	2.85		RPL10A,RPS14,RPS17,RPS19,RPS4Y1
SNARE Signaling Pathway	2.82	1	MYH2,MYH7,MYL3,RAB1A
Peroxisomal protein import	2.81		ACOT2,CAT,CRAT
Electron transport, ATP synthesis, and heat production by uncoupling proteins	2.73	2	CYC1,NDUFB11,NDUFC2,UQCRFS1
Retinoid metabolism and transport	2.71		APOA2,APOA4,TTR
Necroptosis Signaling Pathway	2.61	2	CAPN3,CAPN5,CAPNS1,PPP3CB
Maturity Onset Diabetes of Young (MODY) Signaling	2.52		APOA2,APOA4,APOO
Tight Junction Signaling	2.43		MYH2,MYH7,MYL3,PPP2R1A

Regulation of Cellular Mechanics by Calpain Protease	2.39		CAPN3,CAPN5,CAPNS1
The citric acid (TCA) cycle and respiratory electron transport	2.39		IDH3G,PDK4,SUCLG2
Plasma lipoprotein assembly, remodeling, and clearance	2.38		ALB,APOA2,APOA4
Degradation of beta-catenin by the destruction complex	2.37		PPP2R1A,PSMA6,PSMC1
Glucocorticoid Receptor Signaling	2.31		CYC1,FGG,HP,NDUFB11,PDK4,PPP3CB,UQCRFS1
Hepatic Fibrosis / Hepatic Stellate Cell Activation	2.28		COL1A2,MYH2,MYH7,MYL3
Coronavirus Pathogenesis Pathway	2.23	2	RPS14,RPS17,RPS19,RPS4Y1
Apelin Cardiomyocyte Signaling Pathway	2.21		ATP2A1,CAT,MYL3
Role of Tissue Factor in Cancer	2.2	1	FGA,FGB,FGG,PPP2R1A
Ethanol Degradation IV	2.15		ALDH1A1,CAT
Complement System	2.1		C3,CFH
Serotonin Receptor Signaling	2.08	2.45	FBP2,FGA,FGB,FGG,TGM2,TNNI1

Oxidative Phosphorylation	2.04		CYC1,NDUFB11,UQCRFS1
Signaling by ROBO receptors	2.04		PPP3CB,PSMA6,PSMC1
Multiple Sclerosis Signaling Pathway	2.04	2	C3,CAPN3,CAPN5,CAPNS1
Pathogen Induced Cytokine Storm Signaling Pathway	1.98	1.34	C3,COL1A2,FGA,FGB,FGG
Mitotic G1 phase and G1/S transition	1.93		PPP2R1A,PSMA6,PSMC1
XBP1(S) activates chaperone genes	1.91		ACADVL,HDGF
Intracellular oxygen transport	1.86		MB
Regulation of Apoptosis	1.83		PSMA6,PSMC1
Neutrophil Extracellular Trap Signaling Pathway	1.81	2.24	COL1A2,CYC1,NDUFB11,PPP3CB,UQCRFS1
Detoxification of Reactive Oxygen Species	1.75		CAT,TXNRD1
Microautophagy Signaling Pathway	1.72		COL1A2,PSMA6,PSMC1
FAT10 Signaling Pathway	1.72		PSMA6,PSMC1
Oxytocin Signaling Pathway	1.71	2	MYH2,MYH7,MYL3,PPP3CB
Aryl Hydrocarbon Receptor Signaling	1.7		ALDH1A1,CTSD,TGM2

NIK-->noncanonical NF-kB signaling	1.7		PSMA6,PSMC1
Cellular Effects of Sildenafil (Viagra)	1.7		MYH2,MYH7,MYL3
Inhibition of ARE-Mediated mRNA Degradation Pathway	1.7		PPP2R1A,PSMA6,PSMC1
C-type lectin receptors (CLRs)	1.69		PPP3CB,PSMA6,PSMC1
Pyrophosphate hydrolysis	1.68		PPA1
Thyroid Hormone Biosynthesis	1.68		CTSD
Signaling by Retinoic Acid	1.66		ALDH1A1,PDK4
Mitochondrial protein import	1.63		CYC1,IDH3G
RAB geranylgeranylation	1.62		RAB10,RAB1A
TNFR2 non-canonical NF-kB pathway	1.62		PSMA6,PSMC1
Hedgehog ligand biogenesis	1.59		PSMA6,PSMC1
Mitochondrial Fatty Acid Beta-Oxidation	1.57		ACOT2,ECHS1
Signaling by the B Cell Receptor (BCR)	1.57		PPP3CB,PSMA6,PSMC1
Collagen biosynthesis and modifying enzymes	1.55		COL1A2,SERPINH1

Metabolism of polyamines	1.54		PSMA6,PSMC1
COPII-mediated vesicle transport	1.52		RAB1A,SERPINA1
Thioredoxin Pathway	1.51		TXNRD1
Cellular response to hypoxia	1.5		PSMA6,PSMC1
Regulation of RUNX2 expression and activity	1.5		PSMA6,PSMC1

Supplementary table 6: Top 100 Pathways identified using significant proteins (p<0.05) and the proteins involved in each pathway when comparing the gastrocnemius of NC and C26 mice

Ingenuity Canonical Pathways	-log(p-value)	z-Score	Proteins
Acute Phase Response Signaling	15.7	2.83	APOA2,C3,FGA,FGB,FGG,HP,HPX,ITIH3,ITIH4,SAA1,SERPINA3,SERPINF1,TF,TTR
LXR/RXR Activation	11.4	1.26	APOA2,C3,FGA,HPX,ITIH4,KNG1,SAA1,SERPINF1,TF,TTR
Response to elevated platelet cytosolic Ca2+	11	3.16	ACTN4,FGA,FGB,FGG,ITIH3,ITIH4,KNG1,SERPINA3,TF,WDR1
DHCR24 Signaling Pathway	10.3	1.26	APOA2,C3,FGA,HPX,ITIH4,KNG1,SAA1,SERPINF1,TF,TTR
Intrinsic Prothrombin Activation Pathway	8.6	0.45	COL1A1,COL1A2,FGA,FGB,FGG,KNG1
Post-translational protein phosphorylation	7.55	1.13	APOA2,C3,FGA,FGG,KNG1,P4HB,TF

Regulation of Insulin-like Growth Factor (IGF) transport and uptake by IGFBPs	7.09	1.13	APOA2,C3,FGA,FGG,KNG1,P4HB,TF
Integrin cell surface interactions	6.75	0	COL1A1,COL1A2,COL6A3,FGA,FGB,FGG
Eukaryotic Translation Elongation	6.41	-2.45	EEF2,RPL7A,RPS17,RPS19,RPS4Y1,RPS7
SRP-dependent cotranslational protein targeting to membrane	6	-2.45	RPL7A,RPN1,RPS17,RPS19,RPS4Y1,RPS7
Eukaryotic Translation Initiation	5.75	-2.45	EIF3C,RPL7A,RPS17,RPS19,RPS4Y1,RPS7
Collagen biosynthesis and modifying enzymes	5.63	-2.24	COL1A1,COL1A2,COL6A3,P4HB,SERPINH1
GP6 Signaling Pathway	5.61	0	COL1A1,COL1A2,COL6A3,FGA,FGB,FGG
Coagulation System	5.52	1	FGA,FGB,FGG,KNG1
Glycogen metabolism	5.37	-1	AGL,PHKA1,PHKB,UGP2
GP1b-IX-V activation signalling	5.31		COL1A1,COL1A2,YWHAZ
Microautophagy Signaling Pathway	5.15		COL1A1,COL1A2,COL6A3,PSMA4,PSMA6,PSMD13
Formation of Fibrin Clot (Clotting Cascade)	5.11	2	FGA,FGB,FGG,KNG1
Neutrophil degranulation	5.06	-0.33	AGL,C3,CAT,EEF2,HP,PSMD13,RAB10,SERPINA3,TTR

Eukaryotic Translation Termination	5.01	-2.24	RPL7A,RPS17,RPS19,RPS4Y1,RPS7
Cachexia Signaling Pathway	4.88	1.41	CAPNS1,FGA,FGB,FGG,P4HB,PSMA4,PSMA6,PSMD13
Response of EIF2AK4 (GCN2) to amino acid deficiency	4.88	-2.24	RPL7A,RPS17,RPS19,RPS4Y1,RPS7
Extracellular matrix organization	4.77	-2.24	COL1A1,COL1A2,COL6A3,DCN,TTR
Extrinsic Prothrombin Activation Pathway	4.75		FGA,FGB,FGG
Nonsense-Mediated Decay (NMD)	4.6	-2.24	RPL7A,RPS17,RPS19,RPS4Y1,RPS7
Binding and Uptake of Ligands by Scavenger Receptors	4.37	0.45	COL1A1,COL1A2,HP,HPX,SAA1
TR/RXR Activation	4.33	0.45	ATP2A1,COL6A3,FGA,HP,MBP
Hedgehog ligand biogenesis	4.28	0	P4HB,PSMA4,PSMA6,PSMD13
EIF2 Signaling	4.24		EIF3C,RPL7A,RPS17,RPS19,RPS4Y1,RPS7
Selenoamino acid metabolism	4.09	-2.24	RPL7A,RPS17,RPS19,RPS4Y1,RPS7
Regulation of TLR by endogenous ligand	4.02		FGA,FGB,FGG

Pathogen Induced Cytokine Storm Signaling Pathway	4	0.38	C3,COL1A1,COL1A2,COL6A3,FGA,FGB,FGG
Signaling by NOTCH4	3.96	1	PSMA4,PSMA6,PSMD13,YWHAZ
IL-17A Signaling in Fibroblasts	3.94	-2	COL1A1,COL1A2,P4HB,SERPINH1
Class I MHC mediated antigen processing and presentation	3.9	1.13	FGA,FGB,FGG,PSMA4,PSMA6,PSMD13,SAR1B
RAF/MAP kinase cascade	3.84	1.63	FGA,FGB,FGG,PSMA4,PSMA6,PSMD13
Integrin signaling	3.75		FGA,FGB,FGG
Major pathway of rRNA processing in the nucleolus and cytosol	3.7	-2.24	RPL7A,RPS17,RPS19,RPS4Y1,RPS7
Striated Muscle Contraction	3.68		DES,MYBPC2,TCAP
Regulation of eIF4 and p70S6K Signaling	3.67		EIF3C,RPS17,RPS19,RPS4Y1,RPS7
Collagen chain trimerization	3.56		COL1A1,COL1A2,COL6A3
Coronavirus Pathogenesis Pathway	3.52	2.24	KNG1,RPS17,RPS19,RPS4Y1,RPS7
MyD88:MAL(TIRAP) cascade initiated on	3.5		FGA,FGB,FGG

plasma membrane			
mTOR Signaling	3.37		EIF3C,RPS17,RPS19,RPS4Y1,RPS7
Regulation of Apoptosis	3.32		PSMA4,PSMA6,PSMD13
FAT10 Signaling Pathway	3.16		PSMA4,PSMA6,PSMD13
NIK--> noncanonical NF-kB signaling	3.12		PSMA4,PSMA6,PSMD13
Assembly of collagen fibrils and other multimeric structures	3.06		COL1A1,COL1A2,COL6A3
Collagen degradation	3.06		COL1A1,COL1A2,COL6A3
Platelet Adhesion to exposed collagen	3.02		COL1A1,COL1A2
TNFR2 non-canonical NF-kB pathway	3		PSMA4,PSMA6,PSMD13
Platelet Aggregation (Plug Formation)	2.97		COL1A1,COL1A2
Inhibition of ARE-Mediated mRNA Degradation Pathway	2.9		PSMA4,PSMA6,PSMD13,YWHAZ
Metabolism of polyamines	2.88		PSMA4,PSMA6,PSMD13
Amyloid fiber formation	2.82		FGA,SAA1,TTR

Cellular response to hypoxia	2.82		PSMA4,PSMA6,PSMD13
Regulation of RUNX2 expression and activity	2.82		PSMA4,PSMA6,PSMD13
BAG2 Signaling Pathway	2.7		PSMA4,PSMA6,PSMD13
Hedgehog 'on' state	2.68		PSMA4,PSMA6,PSMD13
Regulation of mitotic cell cycle	2.64		PSMA4,PSMA6,PSMD13
Plasma lipoprotein assembly, remodeling, and clearance	2.63		APOA2,P4HB,SAR1B
Degradation of beta-catenin by the destruction complex	2.61		PSMA4,PSMA6,PSMD13
KEAP1-NFE2L2 pathway	2.59		PSMA4,PSMA6,PSMD13
Hepatic Fibrosis / Hepatic Stellate Cell Activation	2.59		COL1A1,COL1A2,COL6A3,MYH1
Apelin Liver Signaling Pathway	2.57		COL1A1,COL1A2
MAPK6/MAPK4 signaling	2.57		PSMA4,PSMA6,PSMD13
Role of Tissue Factor in Cancer	2.51	1	FGA,FGB,FGG,P4HB
TCF dependent signaling in response to WNT	2.51	1	PSMA4,PSMA6,PSMD13,YWHAZ

Transcriptional regulation by RUNX3	2.5		PSMA4,PSMA6,PSMD13
Syndecan interactions	2.5		COL1A1,COL1A2
S Phase	2.46		PSMA4,PSMA6,PSMD13
DNA Replication Pre-Initiation	2.45		PSMA4,PSMA6,PSMD13
ABC-family proteins mediated transport	2.33		PSMA4,PSMA6,PSMD13
Hedgehog 'off' state	2.31		PSMA4,PSMA6,PSMD13
Signaling by ROBO receptors	2.28		PSMA4,PSMA6,PSMD13
IL-12 Signaling and Production in Macrophages	2.24	-1	APOA2,C3,COL1A1,COL1A2
Neddylation	2.24	0	COPS7A,PSMA4,PSMA6,PSMD13
Interleukin-1 family signaling	2.21		PSMA4,PSMA6,PSMD13
Synthesis of DNA	2.21		PSMA4,PSMA6,PSMD13
GPVI-mediated activation cascade	2.17		COL1A1,COL1A2
Atherosclerosis Signaling	2.17		APOA2,COL1A1,COL1A2
Mitotic G1 phase and G1/S transition	2.17		PSMA4,PSMA6,PSMD13
TCR signaling	2.16		PSMA4,PSMA6,PSMD13
Cell Cycle Checkpoints	2.09	1	PSMA4,PSMA6,PSMD13,YWHAZ

Iron homeostasis signaling pathway	2.02		HP,HPX,TF
Huntington's Disease Signaling	2		CAPNS1,PSMA4,PSMA6,PSMD13
Transcriptional regulation by RUNX1	1.99		PSMA4,PSMA6,PSMD13
PTEN Regulation	1.97		PSMA4,PSMA6,PSMD13
Senescence Pathway	1.93	1	CAPNS1,CAT,PDK4,SAA1
Detoxification of Reactive Oxygen Species	1.92		CAT,P4HB
C-type lectin receptors (CLRs)	1.92		PSMA4,PSMA6,PSMD13
Degradation of the extracellular matrix	1.8		CAPNS1,DCN
Signaling by the B Cell Receptor (BCR)	1.8		PSMA4,PSMA6,PSMD13
Glycerol-3-phosphate Shuttle	1.77		GPD2
Pyrophosphate hydrolysis	1.77		PPA1
Retinoid metabolism and transport	1.76		APOA2,TTR
Granzyme A Signaling	1.71		MBP,NDUFB11

Production of Nitric Oxide and Reactive Oxygen Species in Macrophages	1.71		APOA2,CAT,PPP1R3A
ILK Signaling	1.68		ACTN4,MYH1,NACA
Translocation of SLC2A4 (GLUT4) to the plasma membrane	1.67		RAB10,YWHAZ

Supplementary table 7: Proteins identified on the HDL of CT, NC, C26-HDL peak 1 and C26-HDL peak 2

CT	NC	C26-HDL peak1	C26-HDL peak 2
A2M	A2M	A2M	A2M
ACTG1	A2MP	A2MP	A2MP
ACTR5	ACTG1	ACTG1	ACTB
AFM	ACTR5	ACTR5	ACTG1
AHSG	AFM	AFM	ACTR5
ALB	AHSG	AHSG	ADSL
AMBP	ALAD	ALAD	AFM
APCS	ALB	ALB	AHSG
APOA1	ALDOA	AMBP	ALB
APOA2	AMBP	APCS	ALDH1A1
APOA4	APCS	APOA1	ALDOA
APOB	APEH	APOA2	AMBP
APOC2	APOA1	APOA4	APCS
APOC3	APOA2	APOB	APOA1
APOC4	APOA4	APOC2	APOA2
APOD	APOB	APOC3	APOA4
APOE	APOC2	APOD	APOB
APOH	APOC3	APOE	APOC2
APOM	APOC4	APOM	APOC3
APON	APOD	APON	APOC4
B2M	APOE	B2M	APOD
C1RA	APOH	BNIP1	APOE
C1S1	APOM	BTD	APOM
C3	APON	C1QC	APON
C4B	B2M	C1RA	C1QB
C5	BLMH	C1S1	C1QC
C7	BNIP1	C3	C1RA
C8A	C1QB	C4B	C1S1

C8B	C1RA	C4BPA	C2
C9	C1S1	C5	C3
CAT	C3	C6	C4B
CES1C	C4B	C7	C5
CFH	C4BPA	C8A	C6
CFHR1	C5	C8B	C7
CFHR2	C6	C8G	C8A
CFI	C7	C9	C8B
CFP	C8A	CAT	C8G
CLU	C8B	CD5L	C9
CP	C8G	CES1C	CA2
CPN1	CAT	CFH	CAND1
CPN2	CD5L	CFHR1	CAT
CSF1R	CDH13	CFI	CD5L
DNAH2	CES1C	CFP	CES1B
ECM1	CFH	CLCA3A1	CES1C
EFEMP1	CFHR1	CLU	CFH
EGFR	CFHR2	CP	CFHR1
F10	CFI	CPB2	CFI
F13B	CFP	CPN1	CLCA3A1
F2	CLU	CPN2	CLU
FBLN1	CP	CSF1R	COL1A1
FCN1	CPN1	ECM1	CP
FGA	CPN2	ECM1	CPB2
FGB	CSF1R	EGFR	CPN1
FGG	DNAH2	F10	CPN2
FN1	ECM1	F13B	CRP
GC	EFEMP1	F2	CSF1R
GM20547	EGFR	F5	CTSC
GM8797	F10	F9	DNAH2
GPLD1	F13B	FBLN1	ECM1
GSN	F2	FGA	ECM1
H2-Q10	F5	FGB	EFEMP1
HBA2	F9	FGG	EGFR
HBB-B1	FBLN1	FGL1	F10
HBB-B2	FCN1	FN1	F13B
HGFAC	FGA	GC	F2
HP	FGB	GM10881	F5
HPX	FGG	GM20547	F7
HRG	FN1	GM4788	F9
HSPA8	GC	GM8797	FBLN1
HYDIN	GDA	GPLD1	FETUB
ICA	GLUL	GSN	FGA
IGH-1A	GM10881	H2-Q10	FGB
IGH-3	GM20547	H6PD	FGG
IGHA	GM4788	HBA2	FN1
IGHG1	GM8797	HBB-B1	GC

IGHG3	GPLD1	HBB-B2	GDA
IGHM	GSN	HGFAC	GM10881
IGHV10-1	H2-Q10	HP	GM20547
IGHV1-18	H6PD	HPX	GM4788
IGHV1-31	HBA2	HRG	GM8797
IGHV2-9-1	HBB-B1	HSPA4	GPLD1
IGHV4-1	HBB-B2	HSPA5	GRN
IGHV5-12	HGFAC	HSPA8	GSN
IGHV8-11	HP	HYDIN	H2-Q10
IGHV8-12	HPX	IGFALS	H6PD
IGKC	HRG	IGH-1A	HBA2
IGKV1-110	HSPA4	IGH-3	HBB-B1
IGKV1-135	HSPA5	IGHA	HBB-B2
IGKV3-7	HSPA8	IGHA	HEXB
IGKV4-61	HVM32	IGHE	HGFAC
IGKV6-13	HYDIN	IGHG1	HP
IGKV6-15	ICA	IGHG1	HPX
IGKV8-28	IGFALS	IGHG3	HRG
IL1RAP	IGFBP3	IGHM	HSPA5
ITIH1	IGH-1A	IGHV10-1	HSPA8
ITIH2	IGH-3	IGHV1-31	HVM32
ITIH3	IGHA	IGHV1-5	HYDIN
ITIH4	IGHE	IGHV2-9-1	ICA
KLKB1	IGHG1	IGHV3-1	IGFALS
KNG1	IGHG1	IGHV4-1	IGFBP3
KNG2	IGHG3	IGHV5-12	IGH-1A
LCAT	IGHM	IGHV7-1	IGH-3
LIFR	IGHV10-1	IGHV7-3	IGHA
LUM	IGHV1-18	IGHV8-11	IGHA
MASP1	IGHV1-31	IGHV8-12	IGHE
MBL1	IGHV1-52	IGHV9-4	IGHG1
MUG1	IGHV2-9-1	IGKC	IGHG1
MUP1	IGHV3-1	IGKV1-135	IGHG2B
NUCB1	IGHV4-1	IGKV3-7	IGHG3
PKM	IGHV5-12	IGKV4-57	IGHM
PLA2G7	IGHV7-1	IGKV6-13	IGHV10-1
PLG	IGHV7-3	IGKV8-28	IGHV1-18
PLTP	IGHV8-11	ITIH1	IGHV1-31
PON1	IGHV8-12	ITIH2	IGHV1-5
POSTN	IGKC	ITIH3	IGHV1-77
PROC	IGKV1-110	ITIH4	IGHV2-6
PROS1	IGKV1-135	KLKB1	IGHV2-9-1
PRSS3B	IGKV12-44	KNG1	IGHV3-1
PSAP	IGKV3-7	KNG2	IGHV4-1
QSOX1	IGKV5-39	KPNB1	IGHV5-12
SAA1	IGKV6-13	LCAT	IGHV6-3
SAA2	IGKV6-15	LIFR	IGHV6-6

SAA4	IGKV7-33	LUM	IGHV7-1
SELL	IGKV8-27	MAN2B1	IGHV7-3
SEPP1	IGKV8-28	MASP1	IGHV8-11
SERPINA10	IGLC1	MASP2	IGHV8-12
SERPINA1A	IL1RAP	MUG1	IGKC
SERPINA1B	ITIH1	NUCB1	IGKV1-110
SERPINA3K	ITIH2	PGLYRP2	IGKV1-135
SERPINA3N	ITIH3	PKLR	IGKV12-41
SERPINA7	ITIH4	PKM	IGKV12-44
SERPINC1	KLKB1	PLA2G7	IGKV14-126
SERPINF2	KNG1	PLG	IGKV3-7
SERPING1	KNG2	PON1	IGKV4-57
SPP2	LAP3	POSTN	IGKV4-63
TF	LCAT	PROC	IGKV4-86
THBS4	LIFR	PROS1	IGKV5-39
VTN	LUM	PROZ	IGKV6-13
ZKSCAN2	MASP1	QSOX1	IGKV8-27
	MASP2	SAA1	IGKV9-124
	ME1	SAA2	IGLC1
	MUG1	SAA4	IL1RAP
	MUP1	SELL	ITIH1
	NAP1L4	SEPP1	ITIH2
	NUCB1	SERPINA10	ITIH3
	PGLYRP2	SERPINA1A	ITIH4
	PKLR	SERPINA1B	ITIH4
	PKM	SERPINA3K	KLKB1
	PLA2G7	SERPINA3N	KNG1
	PLG	SERPINC1	KNG2
	PLTP	SERPINF2	KPNB1
	PON1	SERPING1	KV5AC
	POSTN	SOD3	LCAT
	PRDX2	SPP1	LIFR
	PROC	TF	LUM
	PROZ	VASN	MASP1
	PSAP	VPS52	MASP2
	QSOX1	VTN	MTHFD1
	SAA1	ZKSCAN2	MUG1
	SAA2		NSFL1C
	SAA4		NUCB1
	SEPP1		ORM1
	SERPINA10		PGLYRP2
	SERPINA1A		PKM
	SERPINA1B		PLA2G7
	SERPINA3K		PLG
	SERPINA3N		PLOD1
	SERPINC1		PON1

	SERPINF2		PPP1R14B
	SERPING1		PROC
	SPP1		PROS1
	SPP2		PROZ
	STIP1		QSOX1
	TF		SAA1
	THBS4		SAA2
	VPS13D		SAA4
	VPS52		SEPP1
	VTN		SERPINA10
	XDH		SERPINA1A
	ZKSCAN2		SERPINA1B
			SERPINA3K
			SERPINA3N
			SERPINA7
			SERPINC1
			SERPINF2
			SERPING1
			SOD3
			SPP1
			STIP1
			TF
			TFRC
			UBA1
			VCL
			VPS13D
			VPS52
			VTN
			YWHAZ
			ZKSCAN2

Supplementary table 8: Significant proteins identified on HDL

CT vs C26-HDL peak 1[a]			NC vs C26-HDL peak 1[a]			NC vs CT[b]			C26-HDL peak 1 vs C26-HDL peak 2[c]		
Proteins	T-test Difference	p-value	Proteins	T-test Difference	p-value	Proteins	T-test Difference	p-value	Proteins	T-test Difference	p-value
ITIH3	7.06	0.00002	**A2MP**	7.96	0.00008	**VPS52**	2.92	0.04945	**ICA**	7.98	0.00101
A2MP	6.95	0.00272	**ITIH3**	4.61	0.00003	**HP**	2.6	0.02661	**PPP1R14B**	6.85	0.0003
SPP1	6.12	0.00015	**SPP1**	4.54	0.00002	**MASP2**	2.53	0.02434	**C9**	6.35	0.00098
SAA1	5.27	0.00008	**SAA1**	4.11	0.00001	**ITIH3**	2.46	0.04604	**ACTB**	5.61	0.00015
HP	5.04	0.00288	**HSPA5**	3.83	0.00057	**HBB-B2**	2.27	0.01202	**UBA1**	5.54	0.00032
HBB-B2	4.89	0.00098	**H6PD**	3.57	0.00638	**HBA2**	2.04	0.04106	**C6**	5.34	0.04002
HBA2	4.6	0.00368	**SAA2**	3.16	0.00001	**PKLR**	1.84	0.04499	**CPB2**	5.12	0.03148
AMBP	4.25	0.00008	**PROS1**	2.76	0.01219	**HBB-B1**	1.69	0.00335	**HPX**	4.95	0.00034
VPS52	3.95	0.02074	**AMBP**	2.72	0.00084	**PSAP**	-0.7	0.02559	**ALDOA**	4.69	0.00049
HBB-B1	3.5	0.00153	**SERPINA3N**	2.72	0.00286	**KNG1**	-1	0.03213	**CTSC**	4.42	5.00E-05
APON	3.04	0.01311	**HBB-B2**	2.62	0.01396	**APOM**	-1.46	0.02926	**SERPINA7**	4.42	0.03323
HSPA5	3.04	0.008	**HBA2**	2.56	0.00482	**C8B**	-1.6	0.02855	**GRN**	4.4	0.00067
SAA2	2.7	0.0004	**HP**	2.44	0.00972	**SERPINA3K**	-2.2	0.03674	**IGHG1**	4.35	0.00126
IGHE	2.11	0.01115	**BTD**	2.29	0.00029	**PROS1**	-2.69	0.04526	**GSN**	4.33	0.00225

FGA	1.8	0.00385	**SERPINA10**	2.12	0.00003	**PRSS3B**	-2.78	0.02932	**F2**	4.25	0.00021
BTD	1.56	0.0301	**APON**	2.04	0.02971	**ALDOA**	-2.87	0.03488	**ITIH4**	4.24	0.00995
SAA4	1.56	0.00349	**FGL1**	2.04	0.00259				**ORM1**	4.23	0.01922
SERPINA10	1.38	0.02281	**ITIH4**	1.97	0.0051				**NSFL1C**	4.22	0.00033
APOE	1.24	0.04067	**PLG**	1.92	0.04448				**C8G**	4.12	0.00114
SERPING1	1.2	0.01269	**APCS**	1.88	0.03283				**PGLYRP2**	4.07	0.00011
C1S1	-1.24	0.04807	**KNG2**	1.84	0.00005				**IGHG3**	4.01	0.01149
APOM	-1.33	0.01628	**HBB-B1**	1.8	0.01888				**AFM**	3.96	0.00061
C7	-1.46	0.00294	**FN1**	1.77	0.00451				**CES1B**	3.89	0.00667
LIFR	-1.53	0.02002	**CLCA3A1**	1.67	0.00303				**C2**	3.87	0.00127
LUM	-1.61	0.01315	**CFH**	1.53	0.02308				**SERPINF2**	3.87	0.00012
CSF1R	-1.62	0.0394	**SERPING1**	1.48	0.00114				**DNAH2**	3.83	0.00573
ECM1	-1.71	0.03067	**KPNB1**	1.34	0.02747				**ACTG1**	3.82	0.00692
ALAD	-1.77	0.03621	**FGA**	1.31	0.01619				**GM20547**	3.79	5.00E-05
FCN1	-1.78	0.04326	**CP**	1.17	0.00811				**KNG2**	3.66	9.00E-05
HGFAC	-1.79	0.02009	**SAA4**	1.16	0.04258				**GC**	3.6	4.00E-05
EFEMP1	-1.81	0.01112	**FGG**	1.11	0.01415				**CAT**	3.53	0.00029
APOH	-1.99	0.02866	**APOE**	1.05	0.01186				**IGKV4-86**	3.52	0.0052
POSTN	-2.2	0.01783	**C3**	0.92	0.04623				**CA2**	3.51	0.03256
PSAP	-2.31	0.0065	**TF**	0.91	0.0009				**KV5AC**	3.5	0.00258
IGKV6-15	-2.76	0.03508	**APOB**	0.89	0.02387				**HVM32**	3.47	0.01196
PLTP	-2.89	0.00234	**APOD**	-0.76	0.03374				**SERPINA3K**	3.36	0.00067
PROC	-3.15	0.01775	**MASP1**	-0.89	0.00522				**C8B**	3.34	0.00729
IGKV8-28	-3.51	0.04124	**C7**	-1.06	0.00074				**IGHV8-12**	3.22	0.00044
IL1RAP	-3.81	0.01832	**HGFAC**	-1.09	0.01739				**IGLC1**	3.21	0.00525
MUP1	-3.82	0.00099	**IGHV5-12**	-1.21	0.03172				**COL1A1**	3.21	0.00492
IGHG3	-4.05	0.01559	**IGHV1-31**	-1.23	0.02346				**STIP1**	3.2	0.00171
IGHV1-18	-4.12	0.01766	**IGFBP3**	-1.42	0.04201				**IGKV12-41**	3.19	0.01481
DNAH2	-4.13	0.03628	**IGHV2-9-1**	-1.43	0.04158				**CLCA3A1**	3.19	0.00E+00
SPP2	-4.41	0.03585	**LUM**	-1.47	0.00015				**IGHV3-1**	3.15	0.00206
C9	-4.55	0.0239	**LIFR**	-1.51	0.00271				**F9**	3.11	0.01924
ICA	-4.91	0.04593	**IGKC**	-1.61	0.00052				**GDA**	3.09	0.00369
			IGHV1-52	-1.73	0.03221				**IL1RAP**	3.06	0.02981
			PKLR	-1.74	0.01081				**F7**	3.06	0.02207
			IGHV7-3	-1.77	0.0058				**CRP**	2.97	0.0363
			IGHV8-12	-1.78	0.01455				**IGHV6-3**	2.96	0.00714
			EFEMP1	-1.82	0.00201				**CES1C**	2.96	0.00323
			ALAD	-1.83	0.02637				**GM10881**	2.95	0.00342
			APOH	-1.91	0.04122				**IGHV1-18**	2.93	0.00147
			B2M	-2	0.02395				**IGKV14-126**	2.91	0.0108
			PSAP	-2.1	0.01758				**EFEMP1**	2.75	0.00037
			IGKV1-110	-2.17	0.02789				**AHSG**	2.7	0.02273
			CAT	-2.21	0.02088				**VTN**	2.69	0.00024
			IGKV6-13	-2.23	0.02977				**IGHV1-77**	2.57	0.01802
			XDH	-2.26	0.00789				**IGHV10-1**	2.56	0.00549
			DNAH2	-2.3	0.02634				**C8A**	2.52	0.00149
			IGKV8-28	-2.51	0.00707				**IGHG1**	2.51	0.00472
			CDH13	-2.6	0.00002				**IGHV1-5**	2.37	0.02289
			IGHV1-18	-2.75	0.02227				**IGHA**	2.31	0.02395
			MUP1	-2.83	0.00558				**IGH-1A**	2.26	0.0024
			ME1	-2.83	0.01549				**IGHV7-1**	2.23	0.02109
			IL1RAP	-2.92	0.0012				**TF**	2.18	0.01928
			IGKV12-44	-3.37	0.00917				**IGHV8-11**	2.17	0.03263
			SPP2	-3.49	0.0122				**CP**	2.17	0.00117
			IGKV6-15	-3.62	0.00353				**IGKV4-57**	2.16	0.00098
			LAP3	-3.79	0.00033				**YWHAZ**	2.14	0.00089

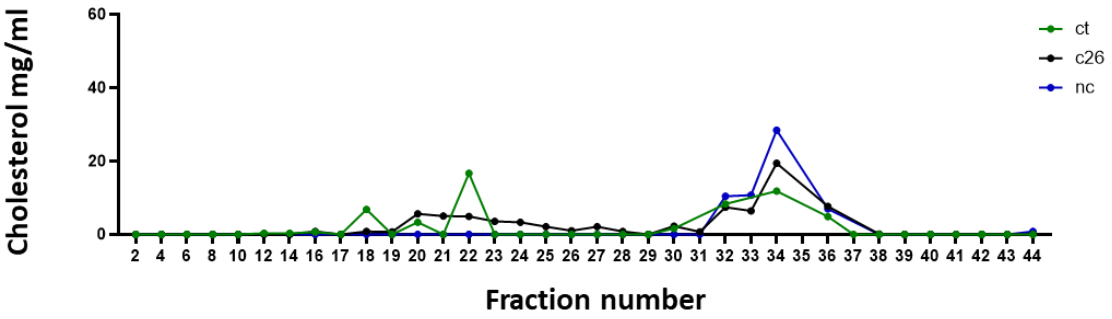
			H2-Q10	-3.8	0.01659				**CLU**	2.1	0.00675
			GDA	-4.34	0.00011				**F5**	2.08	0.04049
									HEXB	2.06	0.00047
									IGFBP3	2.03	0.01288
									IGHV1-31	1.91	0.01274
									IGHV7-3	1.74	0.03891
									IGH-3	1.63	0.01466
									EGFR	1.6	0.00025
									VPS52	1.36	0.03058
									F10	1.17	0.005
									LUM	0.86	0.02425
									SAA4	0.77	0.04736
									ITIH3	-0.83	0.02664
									C1S1	-0.94	0.02481
									VASN	-1.22	0.03023
									APOA2	-1.53	0.01676
									GM4788	-1.56	0.04658
									FBLN1	-1.67	0.02376
									CFH	-1.85	0.00779
									MUG1	-1.86	6.00E-05
									HBB-B1	-1.98	0.00936
									NUCB1	-2.04	0.00818
									POSTN	-2.05	0.00845
									A2M	-2.09	0.01095
									APOE	-2.13	0.00018
									KLKB1	-2.2	0.00261
									HBA2	-2.21	0.00834
									HBB-B2	-2.26	0.04005
									ITIH2	-2.34	0.00163
									CSF1R	-2.44	0.01309
									CPN1	-2.6	0.03346
									HP	-2.64	0.00442
									CFP	-2.81	0.00087
									LIFR	-3.02	0.00453
									SPP1	-3.77	1.00E-05
									CPN2	-5.51	3.00E-05

Significant proteins as determined by t-test ($p<0.05$) in Perseus. [a] =t-test difference indicates proteins up/down regulated in C26-HDL peak 1, [b] =t-test difference indicates proteins up/down regulated in NC, [c] =t-test difference indicates proteins up/down regulated in C26-HDL peak 2.

Supplementary Table 9: Proteins identified on the LDL of CT, NC-LDL peak 1, NC-LDL peak 2, C26-LDL peak 1 and C26-LDL peak 2

CT	NC-LDL peak 1	C26-LDL peak 1	NC-LDL peak 2	C26-LDL peak 2
A2M	A2M	A2M	A2M	A2M
ALB	ALB	ABCA12	ADIPOQ	ABCA12
APOB	APOE	ADIPOQ	AKAP8	ADIPOQ
APOE	FGA	AKAP8	ALB	ALB
C4A	FGB	ALB	APOB	APOB
FGA	FGG	ANKRD18A	APOE	APOE
FGB	FN1	APOB	C3	BRCA2
FGG	HBA1	APOE	C4A	C3
FN1	HBB	BRCA2	FGA	C4A
HBA1	IGKV A18	C3	FGB	FGA
HBB	KRT1	C4A	FGG	FGB

HP	KRT10	CAST	FN1	FGG
IGKV A18	KRT2	FGA	HBA1	FN1
KRT1	KRT9	FGB	HBB	HBA1
KRT10		FGG	HP	HBB
KRT2		FN1	HSPA8	HP
KRT5		HBA1	IGHV1-45	HSPA5
KRT9		HBB	IGKV A18	IGKV A18
MGAM		HP	KRT1	IGKV4-1
PSMA1		HSPA5	KRT10	ITIH4
PSMA2		IGHM	KRT2	KRT1
PSMA4		IGHV1-45	KRT9	KRT10
PSMA5		IGKV A18	MGAM	KRT2
PSMA6		IGKV4-1	PSMA1	KRT9
PSMA7		ITIH4	PSMA2	MGAM
PSMB2		KRT1	PSMA3	PSMA1
PSMB4		KRT10	PSMA4	PSMA2
PSMB5		KRT2	PSMA5	PSMA3
VCP		KRT85	PSMA6	PSMA4
		KRT9	PSMA7	PSMA6
		PSMB4	PSMB1	PSMA7
			PSMB2	PSMB1
			PSMB3	PSMB2
			PSMB4	PSMB3
			PSMB5	PSMB4
			VCP	PSMB5
			ZNF595	SERPING1
				SPARCL1
				VCP
				ZNF595



Supplementary figure 1: Cholesterol measured per FPLC fraction.

Chapter 5:

General discussion

Background:

The primary objective of this thesis was to investigate the utility of the High Density Lipoprotein (HDL) proteome as a biomarker of cancer cachexia and sarcopenia in patients with gastrointestinal cancer. Cancer cachexia is a multi-organ[610], inflammatory[60] and metabolic disorder[611] culminating in the loss of muscle with or without fat loss in patients with cancer[2]. It affects up to 80% of patients with cancer[1], resulting in greater treatment toxicity[24] and surgical complications[29], reduced survival[575, 576] and, importantly, reduced quality of life[574]. Despite its prevalence, cachexia is largely unaddressed in the clinical setting[577, 578], with the majority of patients not receiving cachexia screening or nutrition advice[58]. While this may be due to lack of available treatment options[33], other hurdles include lack of awareness among healthcare professionals[33], lack of a standardised definition[33], reliance on prior weight history and the cost and resources to adequately screen patients[33, 48]. This is a particularly large barrier in the identification of cachexia as, ideally, patients would be monitored over the course of their care. Adding to the complexity of this disease, is the presence of sarcopenia, the loss of muscle strength and mass loss acquired over a life-time[9]. As patients with cancer are typically older, they may have began their cancer journey with sarcopenia[11-13] or developed it as a side effect of the disease. However, as people are not routinely screened for muscle mass, it is impossible to know if sarcopenia within the cancer setting is caused by age, disease or most likely a combination of both. Furthermore, sarcopenia may go unnoticed in patients with obesity, potentially putting them at greater risk of complications[730].

A biomarker that can be integrated into routine care would greatly improve the diagnostic rates for cachexia. However, as outlined in chapter 1, the search for a biomarker is still ongoing. Many of the proposed biomarkers, such as CRP, IL-6 and GDF-15 lack specificity for cachexia and can be affected by a host of other disorders and life-style factors. Many also face a reproducibility crisis, as is the case for IL-6, which has long been associated with cachexia, however has not been consistently identified as a biomarker[182, 183]. Others face practical challenges such as TNFα which has low bioavailability[71], a short half-life[71] and can be affected by collection methods[72]. Some newer approaches are moving beyond the one protein biomarker strategy and creating “scores” for the diagnosis of the disease such as the modified Glasgow Prognostic Score (mGPS) which combines CRP and albumin[158]. However this has also not been adapted for clinical use. Exosomes, lipid bilayer micro-vesicles containing a complex mixture of proteins, micro-RNA, and lipids, are currently under investigation for their biomarker potential in cachexia[455]. These vesicles act as messengers to other cells and tumour derived exosomes have been shown to induce muscle and adipose tissue loss in cancer[731]. However, conversely, these vesicles run the risk of being too specific and representative more of the tumour type rather than as a marker of cachexia[455].

In this thesis, we used a novel approach, the HDL proteome, to explore potential biomarkers of cachexia. HDL are protein rich lipoprotein particles[710], derived primarily from the liver[613] and characterised by their abundant apolipoprotein A1 (ApoA1) content[732]. They range in size from small to large (<9.8nm-16.5nm)[733], carry ~285 proteins[223] and have various functions, most famously reverse cholesterol transport (RCT), but also anti-inflammatory, and anti-oxidative function[300]. Previously, much of the research focused on the relationship between HDL-cholesterol (HDL-C) levels and cardiovascular risk[734]. However, due to the lack of success with HDL-C raising therapies[734], current research is taking a quality, not quantity, approach to HDL research[735]. HDL can become dysfunctional (poor quality) in response to disease and inflammation through modulation of the proteome and lipidome[736]. For example, HDL becomes dysfunctional after the induction of the acute phase response (APR), with increases in serum amyloid A (SAA) and ceruloplasmin (CP) and decreases in ApoA1, the anti-oxidant protein paraoxonase 1 (PON1) and platelet activating factor acetylhydrolase (PAF-AH)[227]. The HDL proteome is also modulated in response to various conditions such as obesity[376, 570, 625], cardiovascular disease[226, 298, 620-622] and diabetes[224, 623, 624], with unique signatures throughout. It is this response to inflammation and metabolic disturbances that led to the hypothesis that the HDL proteome may be affected by cachexia and sarcopenia and act as a biomarker. To investigate this hypothesis, we first investigated the utility of the HDL proteome as a marker of sarcopenia risk in a cohort of participants that were involved in the Nutrimal study, a study that investigated the benefits of leucine ± n-3 LC PUFA on muscle mass and strength in older people (>65 yrs)[289]. This also allowed us to investigate the response of the HDL proteome to changes in diet. We then investigated the utility of the HDL proteome as a biomarker of cachexia and sarcopenia in gastrointestinal cancer patients, combining multiple proteins into a score to create a unique marker of disease. Lastly, we supported some of our human biomarkers with results from a C26 mouse model of cachexia by comparing the HDL of mice with cachexia (C26), to mice with cancer but not cachexia (NC) and control mice without cancer (CT). Using proteomics, this last study also allowed us to investigate changes in the liver that may lead to the development of cachexia. It also allowed us to take a closer look at the relationship between the HDL proteome and pathways affected in the liver.

Summary of study 1-Modification of the protein cargo of HDL particles in patients at risk of sarcopenia – a novel sensor to guide precision nutrition interventions:

Study 1 was not within the original research plan for this thesis, however, due to delays caused by Covid-19, we could not access the gastrointestinal cancer cohort samples. This became an excellent opportunity for us to explore the ability of the HDL proteome to reflect measures of strength and muscle loss, in a non-cancer cohort. Using small (S) and large (L) HDL, this study confirmed that the HDL proteome can act as a biomarker of sarcopenia risk (moderate- vs high-risk) in participants enrolled

in the Nutrimal study[289]. Some of these biomarkers include prothrombin (F2), ceruloplasmin (CP), beta-ala-his-dipeptidase (CNDP1) and serum paraoxonase 1 (PON1). Study 1 also showed a strong correlation between the HDL proteome and handgrip- and leg-strength and skeletal muscle index (SMI). These correlations highlighted potential pathways contributing to strength and muscle loss such as inflammation (Ceruloplasmin (CP), alpha-2-HS-glycoprotein (AHSG), alpha-2-macroglobulin (A2M) and SAA2-SAA4), oxidative stress (Apolipoprotein D (ApoD)), complement activation (complement C3 (C3), complement C5 (C5), complement C6 (C6)) and tissue injury response (gelsolin (GSN), hepatocyte growth factor activator (HGFAC)). This study also allowed us to investigate the affects of dietary changes on the HDL proteome. The Nutrimal study investigated the effects of a leucine (Leu) ± n-3 long chain (LC) polyunsaturated fatty acids (PUFA) on strength, muscle mass and functionality, however it did not find improvements in response to supplementation[289]. Despite this, study 1 showed that the S- and L-HDL proteome were greatly affected by supplementation, including the maltodextrin control, with unique changes in response to each. Apparent beneficial changes in response to Leu + LC n-3 PUFA on L-HDL include decreases in the marker of tissue injury, actin, cytoplasmic-1, the inflammatory marker SAA2-SAA4 and complement proteins (complement C7 (C7) and complement C1s subcomponent (C1S)). Leu + LC n-3 PUFA also uniquely affected several apolipoproteins on L-HDL, reflective of n-3 PUFAS actions on triglycerides. In summary, the HDL proteome is a novel biomarker of sarcopenia risk in healthy, community dwelling, older adults (>65 yrs) and a potential marker of muscle mass and strength. We have also shown that the HDL proteome responds to dietary changes, reflecting potential benefits that are not yet evident in strength and muscle mass. Study 1 supports the hypothesis that the HDL proteome may be a biomarker of the inflammatory-metabolic disorder, cancer cachexia.

<u>Summary of study 2-High Density Lipoprotein proteomic signatures as potential biomarkers of cachexia and sarcopenia in a cohort with gastrointestinal cancer:</u>

In study 2, we demonstrated that the HDL proteome can act as a novel biomarker of cachexia in a gastrointestinal cancer cohort (*n*=84). Using the 2011 Fearon et al., definition, patients were divided into those with cancer cachexia CC (*n*=47) and those without (C-C) (*n*=26). Proteomic analysis of patients HDL showed that *n*=13 proteins were identified when comparing CC to C-C. Decreased proteins included Vitronectin (VTN), CNDP1, Phosphatidylinositol-glycan-specific phospholipase D (GPLD1), Apolipoprotein A-II (ApoA2), HGFAC, Apolipoprotein B (ApoB), Apolipoprotein L1 (ApoL1) and SUN domain-containing protein 3 (SUN3) while increased proteins included Ig Kappa chain C region (IGKC), Thyroxine binding globulin (SERPINA1), Alpha-1-antichymotrypsin (SERPINA3), CP and Vitamin K-dependent protein C (PROC). Using ROC analysis, individual proteins had an area under the curve (AUC) that ranged from 0.637 (SUN3) to 0.720 (SERPINA3), which is considered poor[737]. However when combined into a score, the AUC was 0.810 which is a “considerable” association and clinically useful[737].

This score preformed considerably better than another blood based score, the mGPS, which had an AUC of 0.536 in this cohort. Using the same approach, we created a sarcopenia score comparing cancer patients without cachexia or sarcopenia (C-C) (*n*=26) to patients with cachexia who also met the definition of sarcopenia based on the Martin et al., cut-offs and a small group of patients who had sarcopenia but not cachexia (C+S) (*n*=32). Proteomic analysis of patients HDL identified *n*=16 significant proteins, *n*=7 of which were downregulated, CNDP1, SUN3, ApoA2, GPLD1, ApoL1, Insulin-like growth factor-binding protein 3 (IGFBP3) and Selenoprotein P (SELENOP) and *n*=9 proteins which were upregulated, 78 kDa glucose-regulated protein (HSPA5), SERPINA7, SERPINA3, CP, immunoglobulin Kappa variable 1-8 (IGKV1-8), F2, PROC, Alpha-2-macroglobulin (A2M) and Sex hormone-binding globulin (SHBG). The AUC of individual proteins ranged from 0.637 (CNDP1) to 0.737 (HSPA5) while the combined protein score had an AUC of 0.865. Interestingly, *n*=5 were misdiagnosed by the cachexia or sarcopenia score. Collectively, these patients were more inflamed, had a higher BMI, more adipose tissue and lower SMI, however numbers were too small to conduct meaningful statistics. This potentially highlights that our scores are detecting early stages of cachexia or sarcopenia, well before the patients meet the weight or SMI based metrics. In summary, study 2 has shown that the HDL proteome can act as a novel biomarker of cachexia and sarcopenia in gastrointestinal cancer patients.

Summary of study 3-HDL Proteome Remodelling and Hepatic Alterations in a Mouse Model of Cancer Cachexia:

In study 3, we sought to substantiate our findings from study 2 in a mouse model of cancer cachexia. Using a mouse model of cachexia, provided by Proffessor Laure Bindels in the Université catholique de Louvain, we conducted proteomics on the HDL of C26, NC and CT mice. Suprisingly, there appeared to be a double low density lipoprotein (LDL) peak in the NC and C26 mice and a double HDL peak in C26 mice, possibly indicative of distubances in the lipid profile of the NC and C26 mice. In this study, we confirmed that the HDL proteome is a novel biomarker of cancer cachexia. C26-HDL peak 1 was significantly different from CT and NC mice (*n*=46 and *n*=72 respectively) while comparitively few (*n*=16) were signigicantly different between NC and CT mice. The C26-HDL peak 1 was enriched with several acute phase proteins (SERPINA3n, CP, SAA1,SAA2) had reduced metabolic enzymes (which were absent from the human cohort) (Guanine deaminase (GDA), pyruvate kinase (Pklr)) and modulation of apolipoproteins (ApoE, ApoH, ApoD). In this study, we determined that there was a strong relationship between changes in some proteins on HDL and changes in the liver such as HSPA5, SERPINA10, CP, SAA1, while there was no relationship with other proteins such as ALAD, A2Mp, SERPINA3n. It is important to note that the relationship between these proteins and was only evident when all samples were grouped together, with the excpetion of FN1 which was also significant in the NC group. We also substantiated the cachexia and sarcopenia biomarkers ApoB, CP, HGFAC, HSPA5, IGFBP3, PROC and

SERPINA3n on HDL-peak 1. We also identified several proteins on HDL-peak 2 that were biomarkers of cachexia/sarcopenia in the human cancer cohort (VTN, F2, CP, A2M, IGFBP3, ApoA2 and SERPINA7). However, as C26-HDL peak 2 was only compared to C26-HDL peak 1, this should be interpreted with caution. This study also validated our use of HDL as a biomarker of cachexia compared to another lipoprotein particle, LDL, as these particles contained very few proteins. In this study, we also confirmed that the liver is greatly affected in cachexia, while there were no differences between the NC and CT mice. The most significantly affected hepatic pathways in cachexia included, upregulated protein synthesis, downregulation of xenobiotic metabolism, disturbances in energy production and, as reflected on the HDL proteome, induction of the acute phase response.

Collectively, theses studies show that the HDL proteome is a strong contender for the role of biomarker in cancer cachexia and sarcopenia. While previous biomarkers were representative of one particular facet of the disease, such as inflammation, metabolic disturbances or hormonal changes, HDL has the ability to reflect multiple pathways, many of which overlap with mechanisms contributing to cachexia including inflammation[60], oxidative stress[738] and insulin resistance[5]. It is also noteworthy that some of the HDL associated biomarkers identified in this study have previously been implicated in cachexia or sarcopenia, such as IGFBP3[63] and SERPINA3 (SERPINA3n in mice)[335, 485] and complement C3 (C3) [326, 335] in chapter 2, highlighting that the HDL proteome may be capturing the cachectic/sarcopenic process rather than just general inflammation. The repetition of some biomarkers throughout the study also strengthens our rational for this novel biomarker. For example, CP, an acute phase protein and well-known resident of inflammatory HDL[227] was associated with a high-risk of sarcopenia in study 1, was identified as a biomarker of cachexia and sarcopenia in study 2 and was also found to be a biomarker of cachexia in study 3. Similarly, CNDP1 was identified as a biomarker of sarcopenia risk in study 1 and as a biomarker of cachexia and sarcopenia in study 2. VTN was identified as a biomarker of sarcopenia risk in study 1 and a biomarker of cachexia but not sarcopenia in study 2 while F2 was identified as a marker of sarcopenia risk in study 1 and a biomarker of only sarcopenia in study 2. Interestingly, while IGFBP3 has previously been identified as a biomarker of cachexia[63], it was only a biomarker of sarcopenia in study 2. It was also identified as a marker of handgrip-strength but not SMI at baseline in study 1 while it did not correlate with bodyweight or the weights of liver, tibialis, gastrocnemius, brown adipose tissue or subcutaneous adipose tissue in mice. Previous research has shown that it is involved in both muscle[699] and adipose tissue[63] wasting and so more research is needed to determine the relationship between HDL associated markers and the development of cachexia and sarcopenia. Study 3 allowed us to identify the relationship between HDL associated biomarkers and the liver and showed that while some proteins correlated with levels in the liver, such as the acute phase response proteins SAA2, CP and ITIH4, others did not including the SERPINA3n, serotransferrin (TF) and delta-

aminolevulinic acid dehydratase (ALAD). It would be interesting to further investigate the relationship between these markers and disease to determine if they could be involved in the disease process, or only act as a biomarker.

Limitations

While the studies outlined here provide robust evidence that the HDL proteome is a good biomarker of cachexia and sarcopenia, there are several limitations to consider. In study 1, we lacked a group of participants with fully-developed sarcopenia which would have allowed us to track the effects of disease progression on the HDL proteome, potentially identifying a biomarker of early versus late stage disease. This would be particularly advantageous as treatment options for sarcopenia are limited to dietary and exercise intervention[739-741]. Targeting people in the early stages of the disease, before substantial muscle loss, may make these interventions more effective and prevent further decline. Repeating this study with a larger cohort may also help us to identify biomarkers of responders versus non-responders to the intervention. This would be particularly advantageous in geriatric research as it would help identify people who may not benefit from nutrition intervention alone and may need more aggressive treatment. Additionally, including a group of people with sarcopenia but not cancer in study 2 would have been very beneficial in teasing out the difference between cachexia and sarcopenia. This may have also helped us identify sarcopenia that is driven by the tumour versus sarcopenia of old age, potentially indicating patients that are in need of more urgent attention. Another limitation of this study is that men and women were combined for analysis, due to the small study number. This is particularly important to rectify in future research as women have higher levels of HDL-C, lower LDL-cholesterol and triglycerides and larger HDL particle size[742]. As the protein cargo of S- and L-HDL differs, this may affect potential biomarkers. Additionally, while there has been little research on gender differences in the HDL proteome, previous research in the McGillicuddy group by Dr. Rachel Byrne has shown that the HDL proteome is different between men and women under a number of conditions including obesity and type-II diabetes[743]. Notably, men are more likely to experience cancer cachexia, with potentially different mechanisms driving muscle wasting in men versus women. A “state of the science review” investigating skeletal muscle biopsies in cancer patients found a number of difference in the gene expression of atrophy (Forkhead box O1), autophagy (Cathepsin L2, Beclin 1), inflammation (Janus kinase (JAK) 1, JAK2, Signal transducer and activator of transcription 3 (STAT3)) and muscle growth (myostatin, dystrophin) genes in the skeletal muscle of men and women[744]. Interestingly, women are more susceptible to the non-inflammatory disuse atrophy[745], a consideration for hospital patients who may be less mobile, while the sex prevalence of age-associated sarcopenia is less consistent and changes from study to study[746, 747].

A limitation of study 2 is its cross-sectional nature. With only 1 time-point, we cannot tell how the HDL proteome will evolve with disease progression or if proteins may be predictive of future strength loss. With more time-points, it would have been interesting to see if the patients misdiagnosed by our cachexia or sarcopenia score did go on to develop weight loss or low muscle mass, further supporting the utility of our scores. While most biomarker studies in cachexia have this cross-sectional design, it would be useful to track changes across the course of patients care. This is particularly important given that cachexia can be caused or made worse by anti-cancer treatments[748, 749]. This is a limitation of study 2 as patients are at different stages of their treatment cycle with several treatment regimes within the cohort. Therefore, we are unable to determine if the biomarkers are representative purely of tumour-associated muscle loss or affects of treatment. However, this is representative of a typical cancer cohort and it is important to note that there were no significant differences between the treatment cycle of C-C versus CC and C-S versus C+S. A further limitation is the number of cancer types included in study 2. Patients in this study had colorectal, gastric or oesophageal cancer and so further research is necessary to not only validate the biomarkers in another cohort of patients with gastro-intestinal cancer but also in a cohort of patients with different cancer types. Additionally, as we only used proteomic analysis in this study, validation of the HDL associated biomarkers is needed. A common validation technique in HDL proteomics is western blot[374, 750, 751], however targeted proteomics would also be a valid approach[752].

A limitation of study 3 is the preparation method of the muscle tissue which limited our ability to identify affected pathways and fully evaluate the relationship between the HDL proteome and muscle. We utilised whole muscle tissue which resulted in an uncharacteristically low number of proteins identified, however, previous research by Massart et al., used the sarcoplasmic and myofibrillar fractions in the gastrocnemius of C26 mice and reported far greater yield[335]. This will be an important consideration for future research.

Future directions:

As mentioned above, the first step in developing this research is the validation of identified biomarkers in a cohort of patients with similar cancer types. This may be done using the same methods outlined in this thesis or by developing a targeted proteomic array, as is currently underway in the McGillicuddy group for markers of metabolic health in obesity (MetHealth). This targeted approach, along with a high-throughput HDL isolation method, (also currently being developed in the McGillicuddy group) is key to bringing any potential HDL biomarkers to clinic[617]. The targeted approach is a common validation technique of untargeted proteomics[753] and will enable quicker validation and perhaps refinement of the cachexia and sarcopenia scores. Validation of the identified biomarkers in a cohort of patients with a range of cancer types is also necessary to determine its applicability to a diverse patient population.

While we may overcome the specificity issue associated with other biomarkers with our multi-protein score approach, we also run the risk of developing a biomarker that is specific for cachexia/sarcopenia only in gastro-intestinal cancer patients. Further exploration of the utility of the HDL proteome in identifying cachexia phenotypes/subtypes would be a worthwhile avenue of future research. While we did develop a score to identify patients with low muscle mass (sarcopenia) we did not develop a score to identify patients with low fat mass or low fat mass + low muscle mass. Previously, cachexia has been thought of as one disorder, however, researchers are increasingly recognising that there are multiple phenotypes/subtypes within this disease[754, 755], which may affect outcome. A study by Kays et al., identified *n*=2 cachexia phenotypes within patients with pancreatic cancer undergoing FOLFIRINOX therapy (*n*=53), patients with fat wasting alone (FW) or fat and muscle wasting (FMW), identified using repeated CT-scans[756]. FMW was associated with reduced survival compared to FW (13.0 months and 12.2 months respectively) and compared to patients with no wasting (NW) (22.6 months). Interestingly, in this cohort, 49% of patients had sarcopenia at baseline[756]. The LOTUS CC trial (NCT06073431) is also currently recruiting patients with pancreatic, colorectal or lung cancer with the aim of identifying different cachexia subtypes. This stratification may have implications for future treatment options as, most likely, different cachexia phenotypes have different causes. This would also be particularly useful for clinical trials by identifying the most relevant patients for the treatment outcome. For example, a treatment with the aim of increasing muscle mass would be most suitable for a muscle mass losing phenotype, compared to a fat mass only losing phenotype. This heterogeneity within the cachexia definition may have resulted in the poor cachexia clinical trial results to date. A biomarker that can be used to stratify cachexia phenotypes would not only aid patients in the clinic, allowing for tailoring of treatment options, but also help to identify suitable trial candidates. As we have demonstrated in this thesis, the HDL proteome may reflect different kinds of wasting (different proteins correlated with handgrip- and leg-strength loss and SMI in study 1, and there were key differences in the cachexia and sarcopenia biomarkers in study 2), which would be highly valuable for patient stratification. Additionally, the HDL proteome may have potential to track response to intervention over time and highlight more subtle benefits than improvements in muscle mass and strength. As we have shown in study 1, the HDL proteome is reflective of dietary changes, demonstrating beneficial changes (such as a reduction in complement activation and inflammatory proteins) that were not obvious in more physical markers. It would be an interesting avenue of research to integrate the HDL proteome into a clinical trial and determine if it can reflect changes, identify affected pathways and ideally, identify responders versus non-responders. Previous research has shown that the HDL proteome may be modulated in response to pharmaceutical intervention. In a small study of patients with post-COVID-19 syndrome or post-SARS-CoV-2 vaccination syndrome, treatment with statins and angiotensin II type

1 receptor blockers, improved patients symptoms, increased the expression of FAM3 metabolism-regulating signalling molecule C (FAM3C), ATPase H+ transporting accessory protein 2 (ATP6AP2) and the disintegrin and metalloproteinase domain-containing protein 10 (ADAM10) in the HDL proteome and increased the anti-inflammatory potential of the HDL particle[757]. In another small study (*n*=35), 6 months of metformin increased the expression of peptidoglycan recognition protein 2 (PGRP2) and A2M in the HDL of young people with type-I diabetes[758].

While mechanisms were outside the scope of this thesis, with exception of the liver in study 3, it would be interesting to further investigate the role of HDL in cancer cachexia. While it is known that HDL-C is reduced in cachexia[110, 111], with one study reporting levels similar to those in people with metabolic syndrome[658], not much else is known about the particle during this disease. It is also interesting to note that many HDL functions overlap with processes that contribute to cachexia development, such as inflammation[60, 735], oxidative stress[735, 738], and glucose homeostasis[759, 760]. Additionally, HDL has previously been identified as a biomarker of diabetes[224, 623, 624], a condition that shares some hall-marks of cachexia such as disturbances in the lipid profile[761, 762] and insulin resistance[759, 763]. The relationship between the HDL proteome could be further explored through cell culture based models. It would first be interesting to determine if HDL isolated from patients with cachexia could induce changes in primary human skeletal muscle cells (pHSKM). Secondly, it would be interesting to investigate the effects of atrophying pHSKM (induced by dexamethasone or tumour conditioned media[764]) on a healthy HDL proteome. Furthermore, we could investigate the functionality of the HDL from patients with cachexia through cholesterol efflux assays[765, 766]. In this way we may gain a better understanding of the effects of HDL on the health of skeletal muscle and the effects of the cachectic process on the HDL proteome.

Conclusion:

In summary, we have provided strong evidence that the HDL proteome is a novel, potential biomarker of sarcopenia risk in a non-cancer population, and of cachexia and sarcopenia in a gastrointestinal cancer cohort. We have leveraged a multi-protein approach to overcome the specificity issues that have plagued previously identified biomarkers, providing a circulatory score that could be integrated into routine care. We have also shown that the HDL is highly modifiable by even subtle differences in environment (study 1), opening up future avenues for integration into clinical trials. We have shown some reproducibility throughout our work, from markers of sarcopenia risk in study 1 to markers of cachexia in a C26 mouse model in study 3, however further validation will be needed. We have also added to the growing literature surrounding the role of the liver in cancer cachexia, identifying pathways that may become targets for future treatment.

Chapter 5: Bibliography

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