UNIVERSITAT DE BARCELONA

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Barcelona

2023

Universitat De Barcelona

Facultat de Farmàcia i Ciències de l'Alimentació

Departament de Nutrició, Ciències de l'Alimentació i Gastronomia

Programa de Doctorat

Alimentaciò i Nutrició

Anthocyanins and its microbial metabolites: from food sources to preventing cancer cells migration and improving cardiometabolic health

Thesis presented by Hamza Mohamed Amin Mostafa to obtain Ph.D. degree of University of Barcelon, Spain under the supervision of

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2023

يقول الله تعالى في كتابه الكريم

" وَأَنْزَلْنَا مِنَ السَّمَاءِ مَاءً بِقَدَرٍ فَأَسْكَنَّاهُ فِي الأَرْضِ وَإِنَّا عَلَى ذَهَابٍ بِهِ لَقَادِرُونَ * فَأَنْشَأْنَا لَكُمْ بِهِ جَنَّاتٍ مِنْ نَخِيلٍ وَأَعْنَابٍ لَكُمْ فِيهَا فَوَاكِهُ كَثِيرَةٌ وَمِنْهَا تَأْكُلُونَ *"

القرآن الكريم، سورة المؤمنون ٢٣، الآيات ١٨-١٩

Allah Almighty says in his Holy Book:

“And we have sent down rain from the sky in a measured amount and settled it in the earth, and indeed, we are able to take it away * With it we grow for you gardens of palm trees and grapevines, in which there are abundant fruits, and from which you may eat *”

The Holy Quran, Surat Al Muminun (The Believers) 23, verses 18-19

Alah dice en su libro sagrado:

“Hemos hecho bajar del cielo agua en la cantidad debida y hecho que cale la tierra, y también habríamos sido bien capaces de hacerla desaparecer * Y por medio de ella originamos para vosotros jardines de palmeras y viñedos de los que obtenéis muchos frutos y de los que coméis *”

El Sagrado Corán, Sura Los Creyentes 23, versos 18-19

DEDICATION

To my parents; Hekmat Reihan, Mohamed Salamah, and to my siblings; Arwa, Moaaz, Anas, Baraa and Somaya, and to my nieces and nephews; Layla, Yasin, Hamza, Jumanah and Mariam, I dedicate this work.

إهداء

أهدي هذه الأطروحة لوالديّ الكرام، أمي الحبيبة و قرة عيني حكمت ريحان، أبي سندي و قدوتي محمد سلامة، و إلى أشقائي و شقيقاتي، أروى و معاذ و أنس و براء و سمية، و إلى صغارنا أحباب الرحمن أبناء و بنات أشقائي ليلى و ياسين و حمزة و جمانة و مريم. بفضل الله ثم بفضلكم ودعمكم ودعائكم، ها أنا أتم هذا العمل الجليل، فجزاكم الله عني كل خير.

This work was funded by:

	Agència de Gestió d'Ajuts Universitaris i de Recerca (AGUAR) - Generalitat de Catalunya - 2017SGR1546 / 2021SGR00687
	Insitut de Recerca en Nutrició i Seguretat Alimentària – Universitat de Barcelona Ajuts de personal investigador predoctoral APIF-INSA-UB 2019. Maeztu Unit of Excellence grant (CEX2021-001234-M) funded by (MICIN/AEI/FEDER, UE)
	Centro de Investigación Biomédica en Red Fragilidad y Envejecimiento Saludable (CIBERfes) CB16/10/00269
	DiGuMet project "Diet × gut microbiome-based metabotypes to determine cardio-metabolic risk and tailor intervention strategies for improved health The Joint Programming Initiative A Healthy Diet For A Healty Life (JPI HDHL) Ministerio de Economía, Industria y Competitividad (MINECO) (Spain, PCIN-2017-076)
	FBG 310584 Justus Liebig University Giessen Deutsche Forschungsgemeinschaft
	ICREA Acadèmia 2018

ACKNOWLEDGEMENTS

First and foremost, I would want to give Allah, the Almighty, the God of this world and the hereafter, all the praises and thanks for his blessings and protection throughtout my PhD journey, as well as the Prophet Muhammad (prayers and peace be upon him), the beacon of wisdom and knowledge. Without my faith in Allah, the Almighty, I could never have completed this work.

I am deeply grateful to my parents; Hekmat Reihan and Mohamed Salamah for their love, day and night prayers, sacrifices, concern, and their moral and financial support from the first to the very last day of my PhD, for which I am incredibly grateful. Their support can't be described in words. I also want to express my gratitude to my dear older sister Arwa, for her tremendous scientific guide throughout my PhD and providing me with unwavering support as I pursued my academic goals. Also, I would want to thank my older brothers Moaaz, Anas, Baraa as well as my younger sister Somaya, for their encouragement and support during my study. They have been a constant source of encouragement and have helped me to maintain a positive attitude throughout the challenges of my PhD. I am truly blessed to have such a loving and supportive family, and I am deeply grateful for all that they have done for me.

I would like to express my deep and sincere gratitude to my thesis director, Dr. Cristina Andres-Lacueva for giving me the opportunity to do my PhD in her research group and providing the invaluable guidance, help and support throughout this research. It was an honor to study and work under her supervision. Also, I would like to thank my thesis co-director, Dr. Tomás Meroño for being a friend and an older brother before anything else. I appreciate his constant support, untiring help, guidance, his contribution in the scientific published papers and his motivation throughout my PhD. The happy momeries we shared during these years especially with "El mate" will never be forgotton.

I am incredibly grateful to my lab mates in the Department of Nutrition, Faculty of Pharmacy and Food Sciences, University of Barcelona. Special thanks to Miriam, for her invaluable assistance with the analytical aspects of my research and for her constant support and encouragement. I would also like to extend my thanks to Fabian for being a "Bacan" and for all the English/Spanish conversations we had during our ups and downs the last few years;

Andrea for her friendship and sense of humor which made our lab a fun and welcoming place to work; Nicole for always offering her support and assistance especially with the bureaucratic procedures; Alba for the uncountable Barça matches tickets and for always being present to offer a help during the PhD challenges we had; Blanca for her encouragement messages; and to the new members Meryl, Anna, Montse and Andrea de la Garza for their friendly support and for creating a positive and collaborative environment. I am grateful for the opportunity to have worked with such a talented and dedicated group of individuals, and I am confident that the connections and collaborations formed during my PhD will continue to be beneficial in the future.

It is with immense pleasure that I acknowledge Dr. Sabine Kuntz from Department of Nutritional Science, Justus-Liebig University in Germany. I am grateful for her willingness to collaborate and for her dedication to ATTACH study. Her guidance and feedback have been invaluable in helping me to develop my ideas in cancer cell lines and to bring ATTACH study results and scientific articles to fruition. I would like also to thank her PhD student Inken for her effort and time in ATTACH study publications.

I would like to express my deepest appreciation to DiGuMet project's consortium, for their invaluable guidance, research meetings and scientific input throughout the duration of my PhD and especially in the DCH-NG MAX study. Their expertise and knowledge have appreciated.

I am deeply grateful to Dr. Ana Rodriguez-Mateos, my international research stay's director at Department of Nutrional Sciences, King's College London, for providing me with the opportunity to conduct my research stay at her esteemed institution. Her guidance, expertise, and mentorship have been invaluable, and I feel privileged to have had the opportunity to work under her direction. The insight and feedback she gave me have greatly improved the quality of my research and especially the systematic review. I would like to express my sincere gratitude to her lab students; Alex, Zicheng, Nur Najiah, Julia, Melanie, Ellie, Cambrin, Melis, Yifan, Zoe and Xuemei for their support and assistance during my stay, who have made my research stay an enjoyable, fun and productive experience. Their support, and valuable input have been greatly appreciated.

I would also like to thank Olga Jáuregui from CCiTUB, for her guidance and assistance during the LC-MS analysis which have been a valuable asset, and I am grateful for the opportunity to work under her direction.

I wish to express my sincere appreciation to my ex-lab mates in our Nutrition Departement; Núria for the huge help she gave me during my first months in the lab and for all the support she gave me until the last days of my PhD, Pol for his sense of humor and the fun environment he provided us; Nicola and Magalí for helping me with all the bureaucratic procedures I had to do to apply for the PhD at the group; Gregorio for being a great friend and for his valuable scientific guidance, and finally Alessandra, Gabriele and Julieta for the friendly environment they brought to the lab.

I wish to express my sincere gratitude to Anna Tresserra from our Nutrition Department who has provided me with valuable guidance and support, and for her willingness to share her expertise. Special thanks to her husband Oriol and their lovely kids, Marti and Berta for all the lovely moments we spent together in Belfast and Molins.

I would like to extend my thanks to the other colleagues in our Nutrition Department in Diagonal and Torribera cumpses for their valuable input and for sharing their expertise. I am also grateful for their support and encouragement during the PhD journey.

I am truly thankful to Helena Gaset from Unitat de Beques i Ajuts a l'Estudiant - UB for her invaluable assistance in navigating the bureaucratic procedures associated with my PhD. Her expertise and guidance were essential in helping me navigate the administrative complexities.

I wish to acknowledge the invaluable efforts of Zeinab Hussein in designing the cover of my PhD thesis book and for the other graphical designs I have used during the presentations in my PhD.

I want to extend my sincerest thanks to my friends Amr, Ahmed, Khaled, Khadija, Alexandra, Carla, Sonia, Biagio, Giuseppe, Ayoub, Yasmine, Hinde, Sanam, Mariana, Coni, Fidele, Yasmina, Kamal and to other friends, both near and far, who have provided me with invaluable support and encouragement throughout my PhD journey. Your unwavering friendship has been an essential source of motivation and inspiration.

Finally, I would like to thank my housemates Pilar and David. I am grateful for their friendly support and for creating a positive and supportive environment in the house. I am grateful for the opportunity to have shared the house with such wonderful persons.

With deep gratitude and love,
Hamza Mostafa

ABBREVIATIONS

ACNs	Anthocyanins
ATHENA	AnThocyanin and polyphenols bioactive for Health Enhancement through Nutritional Advancement
ATTACH	Anthocyanins Target Tumor Cell Adhesion-Cancer vs. Endothelial Cell (HUVEC) Interactions Study
CAMs	Cell adhesion molecules
CV	Coefficient of variation
CVD	Cardiovascular diseases
Cya-3-ara	Cyanidin-3-arabinose
DCH-NG MAX	Diet, Cancer and Health-Next Generations Cohort, the MAX study
DiGuMet	Diet x Gut Microbiome-based Metabotypes to determine cardio-metabolic risk and tailor intervention strategies for improved health
3,4-DHPV	3',4'-dihydroxyphenyl-γ-valerolactone
DM	Diabetes Mellitus
DMEM	Dulbecco's modified Eagle's medium
ELISAs	Enzymes linked immunosorbent assays
EPIC	European Prospective Investigation into Cancer and Nutrition
FAK	Focal adhesion kinase
FC	Folin–Ciocalteu
FCS	Fetal calf serum
FOODBALL	Food biomarkers alliance
5-FU	5-Flourouracil
GAE	Gallic acid equivalents
HVAlc	Homovanillyl alcohol
HT-29	Human Colorectal Adenocarcinoma Cell Line
HUVEC	Human Umbilical Vein Endothelial Cells

Mal-3-glc	Malvidin-3-glucoside
MeEC-3-S	3′ -methylepicatechin sulphate
4-Met-Cat-S	4-methylcatechol-sulfate
MFI	Mean fluorescence intensities
MHPV-G	4’-hydroxy-3’-methoxyphenyl-γ-valerolactone glucuronide
MS	Mass Spectrometry
NF-kB	Nuclear Factor kappa B
oCOU	o-Coumaric acid
PAMs	Plasma Isolated Anthocyanins and their Metabolites
PANC-1	Pancreas Ductal Adenocarcinoma Cell Line
PBS	Phosphate buffered saline
Peo-3-gal	Peonidin-3-galactoside
Peo-3-glc	Peonidin-3-glucoside
Pet-3-glc	Petunidin-3-glucoside
ROS	Reactive Oxygen Species
Sal-S	Salsolinol-sulfate
SMD	Standard mean difference
SPE	Solid-Phase Extraction
TEER	Transepithelial electrical resistance
TC	Three-City
THBAld	2,4,6-trihydroxybenzaldehyde
TPC	Total Phenolic Content
UPLC	Ultra-High Performance Liquid Chromatography
UPLC-MS/MS	Ultra-High Performance Liquid Chromatography Coupled To Tandem Mass Spectrometry

VA	Vanillic acid
VEGF-R	Vascular endothelial growth factor-receptor

TABLE OF CONTENTS

ABSTRACT

1. ABSTRACT

Anthocyanins (ACNs) are a type of flavonoid within the family of polyphenols. Plant foods like fruits and vegetables, and particularly berries, are foods rich in ACNs. ACNs are of interest from a health perspective as they may reduce the risk of chronic diseases such as cancer and cognitive diseases, as well as enhance cardiovascular health. This PhD thesis aimed to investigate the effects of anthocyanins (ACNs) and their microbial metabolites on cancer cell migration and cardiometabolic health. The hypothesis was that the positive effects of dietary ACNs on cancer cell migration and cardiometabolic health will be associated with the production of ACN-derived microbial metabolites. The thesis presents four scientific papers; a systematic review and three experimental papers [two from the Anthocyanins Target Tumor Cell Adhesion-Cancer vs. Endothelial Cell (HUVEC) Interactions Study (ATTACH) and one from the Diet, Cancer and Health-Next Generations Cohort (DCH-NG) MAX study)]. The systematic review suggests a multi-metabolite panel, including several gut bacteria metabolites derived from ACNs, as biomarkers of intake for blueberries and cranberries. However, no new multi-metabolite panels were found as biomarkers of intake for raspberries, strawberries, blackcurrants, and blackberries. It also concludes that no specific biomarkers for individual berries exist, and more studies are needed to validate multi-biomarker panels for improving the estimation of berry intake using metabolites concentration in biological samples. In addition, the thesis comprised three original papers with the main objective to investigate the effects of ACNs and their gut bacterial metabolites on colon and pancreatic cancer cells migration and cardiometabolic risk factors. The first paper from ATTACH study demonstrated that plasma extracts after a long-term ACNs-rich juice intervention significantly reduced the migration of colon cancer cells (HT-29) in an in-vitro model. The second paper from ATTACH study showed that from the same plasma extracts also decreased the migration of pancreatic cancer cells (PANC-1) and the expression of cell adhesion molecules via inhibition of the Focal adhesion kinase (FAK) and Nuclear Factor kappa B (NF-kB) pathways, in addition to the reduction of reactive oxygen species (ROS). In addition, metabolomics analyses in plasma and urine identified three plasma metabolites derived from gut bacterial fermentation of ACNs and 11 urine metabolites, including some parent ACNs and gut bacteria metabolites, altered by the ACN-rich juice intervention. The third paper from DCH-NG MAX study demonstrated that the metabolomic

fingerprint of dietary ACNs intake was influenced by their food sources (berries, wine, non-alcoholic drinks, other fruits, vegetables, bakery, dairy products with berries, and mixed dishes) in an observational study. Furthermore, the microbial metabolites linked to berries' ACNs were inversely associated with visceral adipose tissue volume. Overall, this thesis suggests that ACN-rich foods through their microbial metabolites have the potential to prevent cancer cell migration and improve cardiometabolic health. Future randomized controlled trials are warranted to validate the importance of ACN-rich foods for prevention of cancer risk and improve cardiometabolic health and their potential mechanisms of action.

RESUMEN

1. RESUMEN

Las antocianinas (ACNs) son un tipo de flavonoide dentro de la familia de los polifenoles. Los alimentos vegetales como las frutas y verduras, y en particular las bayas, son alimentos ricos en ACNs. Las ACNs son de interés desde el punto de vista de la salud, ya que pueden reducir el riesgo de enfermedades crónicas como el cáncer y las enfermedades cognitivas, así como mejorar la salud cardiovascular. Esta tesis doctoral tuvo como objetivo investigar los efectos de ACNs y sus metabolitos microbianos en la migración de células cancerosas y la salud cardiometabólica. La hipótesis fue que los efectos positivos de ACNs dietéticas en la migración de células cancerosas y la salud cardiometabólica estarán asociados con la producción de metabolitos microbianos derivados de ACNs. La tesis se presenta con cuatro pulicaciones científicas; una revisión sistemática y tres trabajos experimentales (dos del estudio Anthocyanins Target Tumor Cell Adhesion-Cancer vs. Endothelial Cell (HUVEC) Interactions (ATTACH) y uno del estudio Diet, Cancer and Health-Next Generations Cohort, the MAX study (DCH-NG MAX)). La revisión sistemática sugiere un panel de metabolitos múltiples como biomarcador para la ingesta de arándanos y arándanos rojos, pero no se encontraron nuevos candidatos a metabolitos para frambuesas, fresas, grosellas negras y moras. También concluye que no existen biomarcadores específicos para bayas individuales y se necesitan más estudios para validar paneles de biomarcadores múltiples para mejorar la estimación de la ingesta de bayas utilizando la concentración de metabolitos en biofluidos. Además, la tesis comprendió tres trabajos con el objetivo principal de investigar los efectos de ACNs y sus metabolitos bacterianos intestinales en la migración de células cancerosas del colon y páncreas y los factores de riesgo cardiometabólico. El primer trabajo del estudio ATTACH demostró que los extractos plasmáticos después de una intervención de zumo rico en ACN a largo plazo redujeron significativamente la migración de células de cáncer de colon (HT-29) en un modelo in-vitro. El segundo trabajo del estudio ATTACH mostró que ACNs y sus metabolitos extraídos del plasma después de una intervención de zumo rico en ACNs a largo plazo disminuyeron la migración de células de cáncer de páncreas (PANC-1) y la expresión de moléculas de adhesión celular en un modelo in-vitro mediante la inhibición de las vías de la quinasa de adhesión focal (FAK) y el factor nuclear kappa B (NF-kB), además de la reducción de las especies reactivas de oxígeno (ROS). El tercer trabajo del estudio

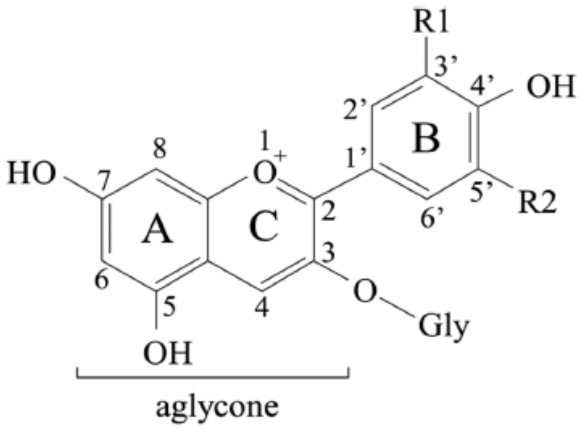
DCH-NG MAX demostró que la huella metabolómica de la ingesta de ACN dietéticas fue influenciada por sus fuentes de alimentos en un estudio observacional. Además, los metabolitos microbianos vinculados a ACNs de las bayas estaban inversamente asociados con el volumen del tejido adiposo visceral. En general, esta tesis sugiere que los alimentos ricos en ACNs y sus metabolitos microbianos tienen el potencial de prevenir la migración de células cancerosas y mejorar la salud cardiometabólica. Se necesitan futuros ensayos controlados aleatorizados para validar la importancia de los alimentos ricos en ACNs en la prevención del riesgo de cáncer y mejorar la salud cardiometabólica y su potencial mecanismo de acción.

INTRODUCTION

2. INTRODUCTION

2.1 Anthocyanins: dietary sources, metabolism, and health benefits

Anthocyanins (ACNs) are water-soluble molecules belonging to the flavonoid class within the large family of organic compounds named polyphenols. ACNs are abundant in plant foods such as berries, grapes, black soybeans, and eggplants, among others. ACNs consist of anthocyanidins attached to glycosyl groups. Anthocyanidins are composed by three aromatic rings, namely the A, B and C ring (Figure 2.1). The A and B rings are benzene rings, while the heterocyclic ring known as the C ring, connects the A and B rings that can have various substituents, such as hydroxyl (-OH) and methoxy (-OCH3) groups (Celli et al. 2017). In nature, up to 700 ACNs can be found in plants as differences at the glycosyl group attached to ring C, and at other substituents of ring B can vary in the basic anthocyanidin structure as shown in Figure 2.1 (Li, Wang, Luo, Zhao, Chen, et al. 2017).



Anthocyanidin (aglycone)	R groups	Approximate distribution in fruits and vegetables
Pelargonidin	R1=R2=H	~ 12%
Cyanidin	R1=OH, R2=H	~ 50%
Delphinidin	R1=R2=OH	~ 12%
Peonidin	R1=OCH_3, R2=H	~ 12%
Petundidin	R1=OH, R2=OCH_3	~ 7%
Malvidin	R1=R2=OCH_3	~ 7%

Figure 2.1. The six most common anthocyanidins. Taken from (Krga and Milenkovic 2019). Gly: glycoside.

ACNs are of interest from a health perspective as they have been linked to different beneficial health outcomes. It has been suggested that ACNs antioxidant and anti-inflammatory properties may reduce the risk of chronic diseases such as cancer and cognitive diseases (Panchal et al. 2022). Additionally, ACNs may improve cardiovascular health and modulate blood glucose levels (García-Conesa et al. 2018; Xu et al. 2021; Panchal et al. 2022). These potential health benefits make dietary ACNs an important target of research for understanding their role in promoting health and preventing chronic diseases through improving human nutrition and the design of tailored dietary interventions.

2.1.1 Dietary sources and intake of ACN in human diet

Studies on human diet suggest that ACNs can be consumed in a variety of ways, from fresh fruits and vegetables to processed foods like juices, jams, wine and bakery, e.g. cakes and other preparations (Zamora-Ros et al. 2011; Lanuza, Zamora-Ros, Rostgaard-Hansen, Tjønneland, et al. 2022). The level of total ACNs intake reported in regular diets ranged from 20-200mg/day in cohort studies. For instance, the mean total ACN intake in a case-control study in Italy to examine the relationship between flavonoid intake and oral and pharyngeal cancer risk was 21.9 mg/day (n=805, 18% of women) (Rossi et al. 2007). In the Diet, Cancer and Health-Next Generations (DCH-NG) MAX study in Danish population (n=624, 55% of women) mean ACN intake was 26.4 mg/day (Lanuza, Zamora-Ros, Rostgaard-Hansen, Tjønneland, et al. 2022). In the European Prospective Investigation into Cancer and Nutrition (EPIC) study (Zamora-Ros et al. 2011) including 10 European countries (n=36,037, 64% of women) mean ACN intake was 23.4 mg/day in the north European region, 29.8 mg/day in the central European region, and 37.4 mg/day in the south European region. Other cohort studies were the Three-Cities (3C) study about polyphenol intake and long term risk of dementia in French population (n=1,329, 62% of women) that reported a mean total ACN intake of 53.9 mg/day (Lefèvre-Arbogast et al. 2018), while it was 161 mg/day in the AnThocyanin and polyphenols bioactive for Health Enhancement through Nutritional Advancement (ATHENA) study in Milano, Italy (n=443, 65% of women) (Rizzi et al. 2016). As it can be seen from the mentioned studies, ACN intake may vary according to dietary habits, geographic location, and other factors such as gender. For example, in the EPIC study,

the total mean ACNs intake for men in Granada, Spain was 38.5 mg/d while for women it was 18.7 mg/d. As the authors reported, the differences in ACN intake between gender in the south European region were due to the high consumption of red wine, which is rich in the ACNs, malvidins. Table 2.1 shows the main food sources of ACNs intake in the European south, central and north regions in the EPIC study.

Table 2.1: Percentage contribution of some of the food items to the intake of total ACNs by European regions. (Adapted from (Zamora-Ros et al. 2011)).

Food items	ACN%		
	North (n=11,764)	**Central (n= 12,988)**	**South (n= 11,285)**
Berries	10.1	16.5	6.3
Other fruits and nuts • Citrus • Apple and pears • Grapes • Other mixed fruits • Olives and nuts	28.0	36.4	54.9
Vegetables • Leafy vegetables • Root vegetables • Cabbages	4.8	8.8	9.7
Wine	24.5	14.4	24.5
Non-alcoholic drinks • Fruit and vegetables juices • Carbonated drinks with berries	19.7	13.6	1.7
Dairy products • With berries as ingredients	0.8	1.5	0.5
Cereal, cakes and confectionery • Fruit cakes • Biscuits with jam and plum cake	4.5	6.5	1.0

2.1.2 Metabolism of ACNs

Upon ingestion of ACN-containing foods, only a small amount (1-2%) of ACNs are absorbed in the stomach and small intestine (Di Lorenzo et al. 2021). In consequence, the majority of ACNs reach the large intestine unaltered where they are metabolized by gut bacteria (Kalt et al. 2020; Igwe et al. 2019; Tian et al. 2019; Kay et al. 2017; Faria et al. 2014; Fang 2014).

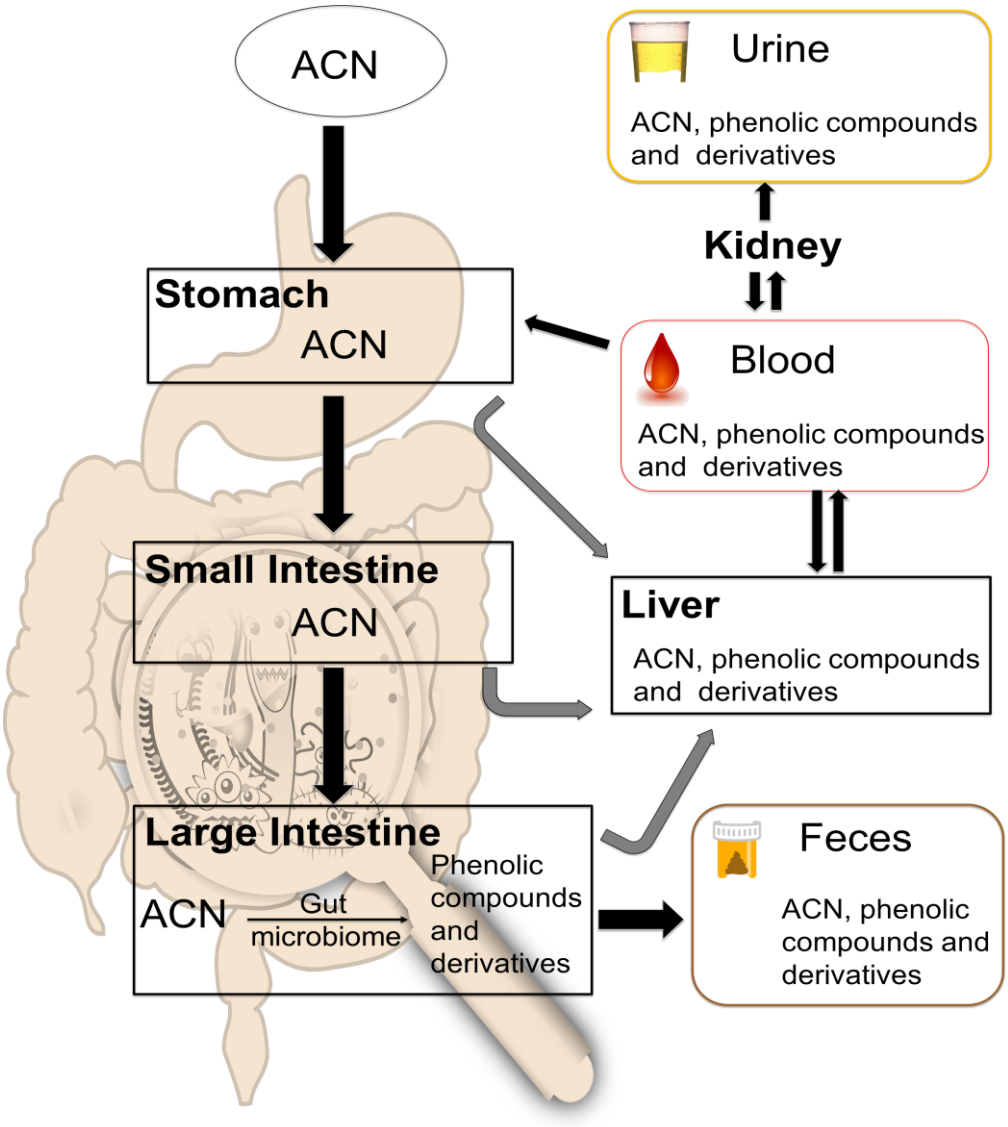


Figure 2.2: Anthocyanins metabolism. Adapted from (Fang 2014; Tian et al. 2019)

The first step in the metabolism of ACNs by gut microbiota is the removal of the sugar moiety from the C-ring. This step produces aglycones, which are then further metabolized through a series of reactions that involve ring cleavage, decarboxylation, demethylation, and reduction, among others (Kay et al. 2017; Tian et al. 2019). These reactions lead to the formation of smaller molecules, mainly phenolic acids as shown in Figure 2.3.

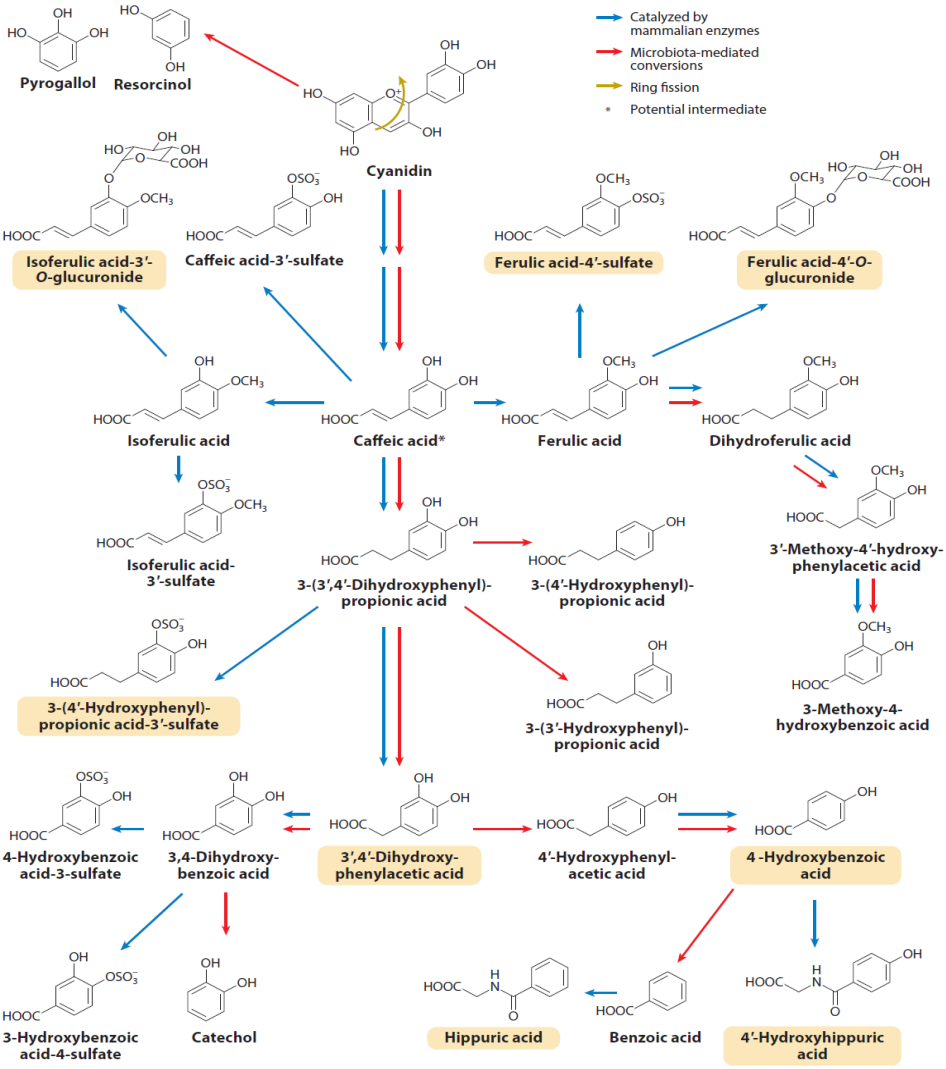


Figure 2.3: Potential pathways for the production of the various phenolic acids after gut microbiota metabolism. Adapted from (Kay et al. 2017).

Several studies have shown that most of the gut bacteria ACN-related phenolic compounds are more bioactive and may reach higher concentrations in circulation than the parent ACNs (Igwe et al. 2019; Tian et al. 2019; Kay et al. 2017; Faria et al. 2014; Fang 2014). Indeed, various phenolic metabolites could be detected in different biological samples (i.e., urine, plasma, etc) after the intake of ACNs. For example, several valerolactones, valeric acid, hippuric, gallic, ferulic and coumaric acids derivatives were found in blood and urine after the consumption of cranberries and raspberries (Favari et al. 2020; Heiss et al. 2022; Zhang et al. 2020). Importantly, these phenolic metabolites have been reported to have several beneficial health outcomes on chronic diseases such as cardiovascular diseases (CVD) and cancer (Heiss et al. 2022; Forester et al. 2014; Augusti et al. 2021). Therefore, gut bacteria-ACNs metabolites might be responsible for at least some of the bioactive effects of dietary ACNs.

Gut microbiota has numerous intra- and inter-individual variabilities during lifespan, and there are various beneficial effects related with the interaction between diet and gut microbiota (Marhuenda-Muñoz et al. 2019). It was stated that there is a two-way interaction between dietary ACNs and gut microbiota that is affecting both the microbial metabolism of ACNs on one hand, and gut bacteria composition, in the other hand (Vendrame et al. 2011).

2.1.3 Scientific evidence linking ACN intake to cancer and cardiometabolic diseases

As discussed in previous clinical trials and reviews, daily intake of ACNs was associated with improved markers of cardiovascular and cognitive health (Li, Wang, Luo, Zhao, and Chen 2017; Rodriguez-mateos 2019; Heiss et al. 2022). Accordingly, epidemiological studies found an association between higher ACNs intake and reduced risk of other chronic diseases such as diabetes mellitus (DM) and cancer (Sun et al. 2021; Parmenter et al. 2021).

Regarding the link between ACNs intake and cancer, a meta-analysis of observational studies including three cohort studies and four case-control studies, with data of more than 500,000 participants, suggested that higher ACN intake may play an active role in the prevention of colorectal cancer ($RR_{\text{highest } vs. \text{ lowest quintile of total ACN intake}} = 0.78$; 95% CI, 0.64–0.95) (Wang et al. 2019). Another meta-analysis including 12 epidemiological studies with more than 2,000

cases and 400,000 non-cases concluded that higher ACN intakes were associated with decreased risk for esophageal cancer (OR $_{\text{highest } vs. \text{ lowest category of total ACN intake}}$ = 0.60, 95% CI: 0.49–0.74; I^2=41.8%) (Cui et al. 2016).

Additionally, in-vitro studies have demonstrated the potential of ACNs to have anticancer activities. For example, a study conducted on human breast and prostatic cancer cells showed that ACN-rich extracts from blueberries inhibited cell proliferation and cell adhesion of cancer cells (Lamdan et al. 2020). Other studies on pancreatic and colon cancer cells found that extracts of ACNs from grapes and berries led to an inhibition of cell migration and proliferation (Pérez-Ortiz et al. 2019; Kuntz, Kunz, and Rudloff 2017). These anticancer effects were attributed to the ability of ACNs to inhibit NF-kB and Matrix Metalloproteinases (MMP), or to downregulate genes involved in cancer development and progression. Nonetheless, up to the moment, most of these studies tested supraphysiological doses of ACNs and whether physiological doses achievable through dietary interventions would display the same effects is not entirely known.

In addition, several studies have shown evidence that ACN intake can be beneficial for cardiometabolic health. For instance, a comprehensive dose–response meta-analysis including 39 prospective cohort studies reported that ACNs intake was inversely associated with CVD (RR $_{\text{highest } vs. \text{ lowest category of total ACN intake}}$ = 0.82, 95% CI: 0.70–0.96; I^2=75%) (Micek et al. 2021). This result was confirmed by other two systematic reviews and meta-analysis (of 24 RCTs and 32 RCTs, respectively) studying the effect of ACNs-rich foods on vascular function (Fairlie-jones et al. 2017; Yang et al. 2017). The first one showed improvement of flow-mediated dilation (FMD, a measure related with endothelial dysfunction and a surrogate marker of subclinical atherosclerosis) following acute supplementation (standard mean difference (SMD)): 3.92%, 95% CI: 1.47, 6.38) or chronic supplementation (SMD: 0.84%, 95% CI: 0.55, 1.12) with ACNs (Fairlie-jones et al. 2017). In addition, they also showed that ACN-supplementation led to a reduction of pulse wave velocity (SMD: -1.27 m/s, 95% CI: -1.96 -0.58), a measurement related with hypertension and increased CVD risk (Fairlie-jones et al. 2017). The second meta-analysis showed improvements in glycemic control by reducing fasting glucose (SMD: -0.31; 95% CI: -0.59, -0.04; I2 = 80.7%), 2h postprandial glucose (SMD: -0.82; 95% CI: -1.49, -0.15), glycated haemoglobin (SMD: -0.65; 95% CI: -

1.00, -0.29), and total cholesterol (SMD: -0.33; 95% CI: -0.62, -0.03), and LDL-C levels (SMD: -0.35; 95% CI: -0.66, -0.05) (Yang et al. 2017). Furthermore, in-vitro studies as well provided evidence that ACNs may have a protective effect on the cardiovascular system. A study reported that phenolic metabolites derived from the gut fermentation of blueberries ACNs showed an antihypertensive effect on human endothelial cells as they caused a higher nitric oxide bioavailability (Bharat et al. 2018). Another study showed that ACNs could inhibit lipid accumulation by regulating adipogenesis and lipogenesis related genes and signaling proteins in human endothelial cells (Imran et al. 2018). Therefore, several lines of evidence suggest that ACNs may have an impact on cardiometabolic health and justify its interest from a nutritional perspective.

2.2 Metabolomics

Metabolomics focuses on the comprehensive analysis, identification and quantification of small molecules with a molecular weight < 1500 Da, metabolites, present in cells, tissues, and living organisms (Rangel-Huerta 2018). Metabolite levels and ratios reflect the metabolic state and can provide insights into various biological processes such as aging, disease development, etc. Each organism's metabolome is often impacted by both internal and external environmental influences (Igwe et al. 2019; Marhuenda-Muñoz et al. 2019). In consequence, changes in the metabolome may be linked to certain diets, lifestyles, use of medications, and diseases (Igwe et al. 2019; Marhuenda-Muñoz et al. 2019). These variations may serve as biomarkers for certain diseases or medical conditions, or as a metabolic fingerprint to decipher potential mechanisms of action. Additionally, it has the potential to build models to anticipate how the body will react to variations in these internal and external factors, which ultimately can help with the identification of early biomarkers of disease, medication discovery, and personalized treatment (Rangel-Huerta 2018; M. M. Ulaszewska et al. 2019).

2.2.1. Nutrimetabolomics: Metabolomics meets nutrition

Nutrimetabolomics is known as nutritional metabolomics and is a specialized area of metabolomics that focuses on the study of the interactions between dietary components and the body's metabolism and how diet influences metabolism and health (M. M. Ulaszewska et al. 2019). By combining the analysis of metabolites with information on the nutrient composition of the food and the physiological status of the individual, nutrimetabolomics has numerous applications in personalized nutrition, development of functional foods, and the design of novel dietary interventions for diseases. Besides, nutrimetabolomics can help to study the complex interplay between diet, and gut microbiota and host metabolism. By analyzing the metabolites in biological samples, the specific dietary components that are being metabolized by the gut microbiota can be identified, as well as the metabolic pathways and end-products that are being produced. This information can help to elucidate the mechanisms by which diet influences metabolism and health and may lead to the identification of new biomarkers or novel therapeutic targets (M. M. Ulaszewska et al. 2019). On the other hand, the composition of the gut microbiota is influenced by a variety of factors, including age, genetics, environment, and diet. Studies have shown that dietary patterns can have a significant impact on the diversity and metabolic activity of gut microbiota (Igwe et al. 2019; Marhuenda-Muñoz et al. 2019). These, in turn, can affect the production and absorption of metabolites in the body, leading to changes in host metabolism and potentially affecting several health outcomes. Different metabolomics platforms may be applied in nutrimetabolomics studies. In the present thesis a targeted, exposome-based metabolomics method was applied.

2.2.2. Main areas of research in nutrimetabolomics

Nutrimetabolomics research has three main areas: i) biomarkers of food intake, ii) metabolomic profiling of diet, and iii) metabotyping. Biomarkers of food intake are specific molecules or metabolites present in biological samples that can be used to measure dietary intake and assess the quality of a person's diet, like for example grapes and berries intake biomarkers (M. Ulaszewska et al. 2020). This research area aims to improve dietary data from self-reported dietary records and questionnaires, which can be inaccurate due to recall

bias or underreporting. Metabolomic profiling of diet involves the comprehensive analysis of metabolites in response to diet (M. M. Ulaszewska et al. 2019). This area allows to investigate the mechanisms underlying the beneficial effects of certain foods, nutrients, or dietary patterns. Last, metabotyping is the clustering of individuals based on common metabolic characteristics based on their unique pattern of metabolites (Palmnäs et al. 2020). Research in this area can be useful for developing personalized nutrition interventions or predicting the response of a group of individuals to a particular dietary intervention. The present thesis focusses in the nutrimetabolomics research area of biomarkers of food intake. An introduction to the fundaments of the targeted metabolomics methodology applied to the study of biomarkers of food intake is described below.

2.2.3. Targeted metabolomics in the study of biomarkers of food intake

Targeted metabolomics is a metabolome profiling approach where the mass spectrum (MS) analysis targets known molecules in order to quantify them with a calibration curve built from authentic standards. Targeted metabolomics is considered specific, sensitive, and accurate, as it involves the development of methods for preparing the biological samples and adjusting the workflow of the instrument to specifically detect and quantify a set of metabolites of interest (Bauermeister et al. 2022). In the context of food metabolome and metabolites derived from gut bacterial fermentation, targeted metabolomics can provide valuable information about the metabolism of these metabolites in the body and their impact on human health by providing precise and accurate measurement of their levels in blood and urine (González-Domínguez et al. 2020). Figure 2.3 shows the human metabolome which can be defined by the sum of external exposures, namely food metabolome, microbiota-derived metabolites, drug-derived metabolites, pollutants and household chemicals, and the endogenous metabolome. The food metabolome has been defined as the entire set of the metabolites directly produced from food digestion, their absorption in the gut, and biotransformation by host tissues and microbiota. Majority of the gut microbiota-derived metabolites are derived from the biotransformation of both the endogenous metabolome and the food metabolome, making them an essential component of these two metabolomes. Short-chain fatty acids, secondary bile acids, protein and amino acid metabolites, and plant

polyphenol metabolites are examples of these microbial metabolites (Scalbert et al. 2014; González-Domínguez et al. 2020). The endogenous metabolome includes all the metabolites from the host besides all the other molecules, such as vitamins, required for an adequate cellular metabolism.

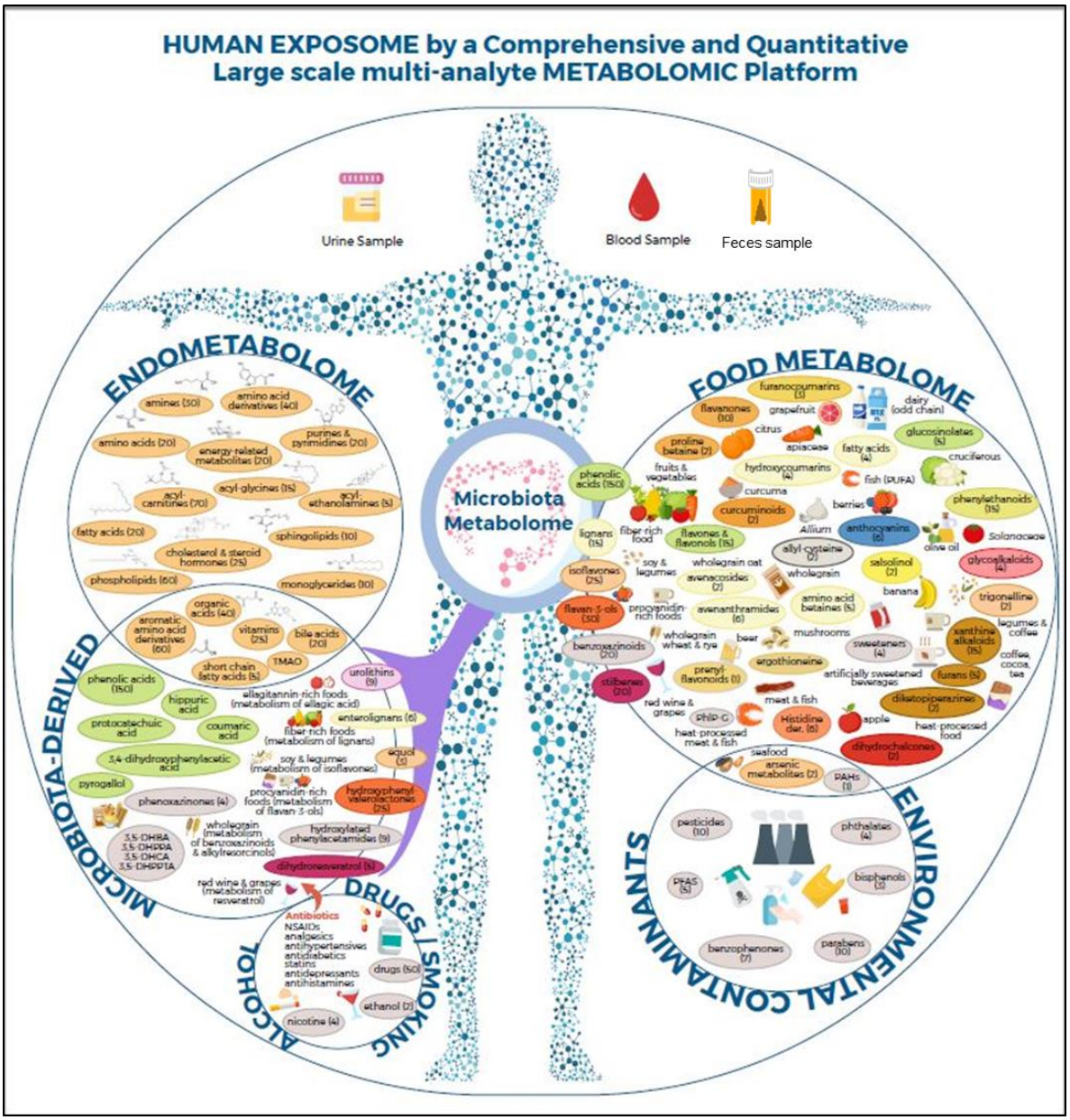


Figure 2.3: Human metabolome used for the targeted metabolomics analysis in this thesis. Taken from (González-Domínguez et al. 2020)

Some of the key challenges in nutrimetabolomics include the large number of metabolites that need to be analyzed in biological samples. However, advancements in the analytical techniques are helping to overcome these challenges. Therefore, nutrimetabolomics is becoming an increasingly important tool for understanding the effects of diet and nutrition on health.

Food intake biomarkers are assumed to provide a more objective reflection of intake than food frequency questionnaires, food diaries and 24h recall methods even though these last represent the most commonly used dietary assessment tools in human studies on nutrition and health. However, although many biomarkers have been described, only relatively few are sufficiently validated and accepted as food intake biomarkers. In the identification of new potential biomarkers of food intake, many factors need to be considered, such as metabolism and lifestyle. Additionally, the specificity of the metabolite for the food under study needs to be evaluated, where also the increasing use of fortified foods and supplements should be considered. By joining international multidisciplinary expertise, the Food Biomarker Alliance (FoodBAll) consortium intended to contribute to the discovery of new food intake biomarkers, using novel metabolomics techniques (Brouwer-Brolsma et al. 2017). Ultimately this effort aims to facilitate three main applications, such as (1) validation/adjustment of self-reported dietary assessment tools, (2) serve as markers of compliance in intervention studies and (3) improve the value of many observational studies investigating nutrition and health associations by providing less biased intake estimates (Brouwer-Brolsma et al. 2017). In addition, the FoodBall consortium has classified dietary biomarkers as indicators to reflect (1) the consumption of food, its compounds or components, or part of a dietary pattern, or (2) the effect or implicated physiological and health status (Noerman et al. 2022).

2.3. ACNs and its gut microbial metabolites as biomarkers of berries intake

Berries are one of the major dietary sources for ACNs in the diet. Moreover, they are included in nutritional recommendations as part of Healthy Diets, such as the healthy Nordic and Mediterranean diets as a source of vitamins, minerals and other polyphenols apart from ACNs (Penttinen et al. 2021; Mithril et al. 2013; Battino et al. 2019). Nonetheless, quantification of ACNs and their gut-related phenolic metabolites and their association with

berry consumption are lacking. Many observational studies that rely on dietary intake assessment methods such as food frequency questionnaires (FFQ) and 24h dietary recalls have the limitation of being subjective methods that provide inadequate quantitative information about the consumption of specific types of foods (Archer, Marlow, and Lavie 2018). Besides, the complexity of the food matrix, especially the fruits that can contain many different common phytochemicals and the variability in the bioavailability of these compounds after consumption can limit the description of diet x health associations. Biomarkers of berries intake have been discussed in a previous systematic review (M. Ulaszewska et al. 2020). Various number of metabolites, including phase II and gut microbial-derived metabolites, have been proposed as biomarker after the intake of berries in previous RCTs. Among them, ellagitannins and their gut microbial metabolites, urolithins, ACNs and its derivatives and some other aromatic compounds (M. Ulaszewska et al. 2020; Favari et al. 2020; Istas et al. 2018). However, because these metabolites are widespread and can be found in a wide range of fruits and vegetables, they aren't suitable for use as singular biomarkers due to a lack of specificity. For this reason, one potential approach is to use a multi-biomarker panel as a biomarker of intake, which has previously been suggested to be a better choice than a single biomarker. However, further studies are in need to discover additional new biomarkers and to improve the previously proposed metabolite panels.

HYPOTHESIS AND OBJECTIVES

3. HYPOTHESIS AND OBJECTIVES

Hypothesis

The positive effects of dietary ACNs on cancer cell migration and cardiometabolic health will be associated with the concentration of ACN-derived microbial metabolites in biological fluids.

Objectives

This thesis has four main objectives:

1. To study the relationship between consumption of berries, which are one of the most relevant sources of dietary ACNs, and metabolome biomarkers. (Publication 1).

2. To test the effects of ACNs and their metabolites isolated from plasma after a 28-days intervention with dietary ACNs on colon and pancreatic cancer cells migration. (Publication 2 and 3).
    a. To detect if plasma-isolated ACNs and their metabolites reduce the migration of two colon (HT-29 and Caco-2) and pancreatic (PANC-1 and AsPC-1) cancer cells in-vitro.
    b. To detect if the expression of cell adhesion molecules (CAMs) on pancreatic cancer and endothelial cells are influenced by plasma extracts after a long-term 28-days intervention with an ACNs rich juice.
    c. To evaluate the implications of the NF-kβ and FAK pathways in the observed effects.

3. To investigate and characterize the changes in plasma, urine, and fecal metabolome after a 28-days intervention with a juice rich in ACNs in healthy subjects. (Publication 3).
    a. To characterize metabolomics alterations in plasma, urine and feces samples before and after 28-days of intervention with an ACNs rich juice vs. an ACN-depleted placebo using a targeted metabolomics method.

   b. To investigate the association between metabolites altered by the ACN-rich juice intervention (in plasma and urine) and the potential of plasma extracts to inhibit cancer cells migration.

4. To assess the relationship between ACNs coming from different dietary sources, metabolome biomarkers, and cardiometabolic risk factors in an observational study. (Publication 4).
   a. To describe total ACNs dietary intake, as well as ACNs main dietary sources in a Danish population.
   b. To study the associations between total ACNs and ACNs coming from different dietary sources with plasma metabolome biomarkers in an observational study.
   c. To investigate the associations between metabolites related with ACNs intake and cardiometabolic risk factors.

METHODOLOGY

4. METHODOLOGY

In this chapter, descriptions of the cell-based in-vitro assays, targeted metabolomics experiments and the statistical analyses conducted in all the publications are briefly detailed.

4.1. Literature search for biomarkers of berries intake

We conducted a systematic review according to the PRISMA statement (Preferred Reporting Items for Systematic Reviews and Meta-Analysis) as detailed in the Table 4.1. Our review employed an extensive search using the following keywords (Fruit name* OR botanical name), AND (urine or plasma or serum or excretion or blood) AND (human* OR men OR women OR patient* OR volunteer* OR participant*) AND (biomarker* OR marker* OR metabolite* OR biokinetics OR biotransformation OR pharmacokinetics OR bioavailability OR ADME) AND (intake OR meal OR diet OR ingestion OR administration OR consumption OR eating OR drink) across three electronic databases: PubMed, Web of Science and Scopus. There was a previous systematic review that has covered published literature until December 2019. To continue upon that, the present systematic search included trials which were conducted only on berries published, in English only, between January 2020 to December 2022. Short- and long-term human interventions studies conducted on berries intake were selected for the review. The main exclusion criteria were animal and in-vitro studies and papers assessed irrelevant diets.

Table 4.1: Keywords used for the systematic search

Database / keywords	(Fruit name* OR botanical name), AND (urine or plasma or serum or excretion or blood) AND (human* OR men OR women OR patient* OR volunteer* OR participant*) AND (biomarker* OR marker* OR metabolite* OR biokinetics OR biotransformation OR pharmacokinetics OR bioavailability OR ADME) AND (intake OR meal OR diet OR ingestion OR administration OR consumption OR eating OR drink*)
PubMed	All fields
Web of Science	Topic
Scopus	Article Title/Abstract/Keywords

4.2. NUTRITIONAL INTERVENTION CLINICAL TRIAL. Anthocyanins Target Tumor Cell Adhesion—Cancer vs. Endothelial Cell (HUVEC) Interactions (ATTACH) study. (Project Code FBG: 310584)

4.2.1. Study design

The ATTACH study (Anthocyanins Target Tumor Cell Adhesion—Cancer vs. Endothelial Cell (HUVEC) Interactions study) is a randomized, placebo-controlled, double blinded, crossover trial which was conducted between April and August 2019 at the Department of Nutritional Science, Justus Liebig University, Giessen (Germany). Thirty-five volunteers (female n = 27 and male n = 8), with a mean (± SD) age of 24 (± 2 years, range: 19–29 years), an initial body weight of 64 ± 18 kg (range: 49–93 kg), and a BMI of 21.7 ± 2.6 kg/m2 (range: 18.4-27.6 kg/m^2) were recruited. Volunteers were randomized into two arms and received the beverages (ACN-rich juice and ACN-depleted placebo), denoted as "one" or "two". The beverages were filled in brown bottles to ensure blinding and distributed to volunteers on a weekly basis. After a 7-days run-in phase in which the volunteers were instructed not to eat any dietary source of polyphenols, for 28 days all the participants were instructed to consume 0.33 L of the drink on a daily basis (Figure 4.1). They were instructed to keep the juices cool and to avoid their exposure to direct light. The first phase of the intervention was completed by a 14-day run-out period. The next phase began with the next beverage after the run-out phase. Prior to the start of the study, participants were explicitly advised to follow a low polyphenols/ACNs diet during the intervention periods to avoid the potential effects of other phenolics, particularly ACNs, from the diet. Blood, urine and fecal samples were collected at day 0 and day 28 of each intervention period. This clinical trial has been registered in the DRKS (Deutsche Register Klinischer Studien) with the registration number DRKS00014767.

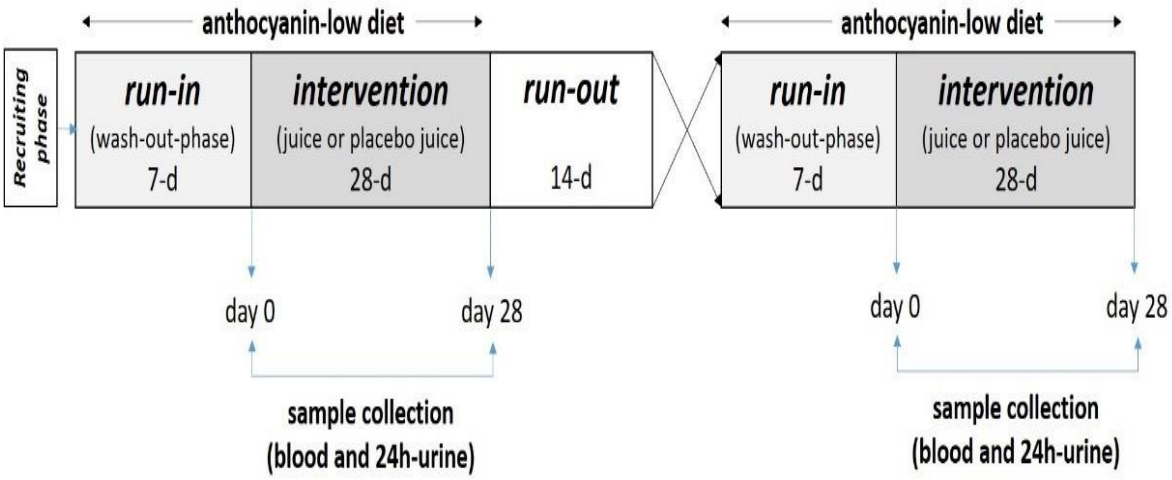


Figure 4.1: Study design of the ATTACH study (Taken from (Behrendt, Röder, Will, Mostafa, Gonzalez-Dominguez, et al. 2022)).

4.1.2. ACN composition of the juices of the intervention

Publication 1 describes the chemical characterization of the ACN-rich juice and the ACN-depleted placebo.

Table 4.2: ACNs composition in the ACN-rich juice and the ACN-depleted placebo (Taken from (Behrendt, Röder, Will, Mostafa, Gonzalez-Dominguez, et al. 2022))

	Anthocyanin-Rich Juice		Anthocyanin-Depleted Placebo	
Anthocyanins	(mg/L)	(%)	mg/L	(%)
peonidin-3,5-O-diglucoside	346 ± 12.5	36.8	1.7 ± 0.02	26.9
malvidin-3,5-O-diglucoside	138 ± 8.4	14.7	0.88 ± 0.06	14.0
peonidin-3-O-glucoside	83.5 ± 6.4	8.9	0.37 ± 0.01	5.9
malvidin-3-O-glucoside	63.4 ± 3.8	6.7	0.30 ± 0.01	4.7
delphinidin-3-O-glucoside	61.5 ± 2.9	6.5	0.80 ± 0.03	12.7
delphinidin-3-O-galactoside	53.6 ± 0.6	5.7	0.75 ± 0.01	11.9
delphinidin-3-O-arabinoside	53.4 ± 1.6	5.7	0.56 ± 0.02	7.4
petunidin-3-O-glucoside	43.7 ± 1.9	4.6	0.37 ± 0.02	5.8
cyanidin-3-O-arabinoside	27.2 ± 1.7	2.9	0.11 ± 0.02	1.7
cyanidin-3,5-O-diglucoside	18.2 ± 1.6	1.9	0.29 ± 0.00	4.6
malvidin-3-(6″-O-coumaryl)-5-O-diglucoside	17.1 ± 0.4	1.8	n.d.	n.d.
petunidin-3-O-galactoside	13.2 ± 0.3	1.4	0.11 ± 0.01	1.8
petunidin-3-O-arabinoside	8.8 ± 0.0	0.9	0.05 ± 0.01	0.8
malvidin-3-O-arabinoside	5.3 ± 0.0	0.6	0.02 ± 0.00	0.3
peonidin-3-O-galactoside	4.3 ± 0.0	0.5	0.01 ± 0.00	0.1
delphinidin-3,5-O-diglucoside	3.4 ± 0.0	0.4	0.09 ± 0.01	1.4
Sum	942 ± 10	100	6.3 ± 0.5	100

The two juices were produced at the Hochschule Geisenheim University (Department of Beverage Research, Geisenheim, Germany) and made from 80% red grape juice (grape variety Accent) and 20% bilberry juice (Heidelbeersaft blank BIO (Bayernwald KG, Hengersberg, Germany)). The grape juice was separated, mixed with the bilberry juice, pasteurized, and hot-filled into 0.33 L brown glass bottles. By passing the juice through SP70 Sepabeads® absorber resin (Resindion S.r.l., Binasco, Italy), placebo juice was obtained. Both juices were analyzed immediately after 0.45 um membrane filtration for basic analytical parameters such as total phenolics and ACNs concentrations. ACNs were determined using LC-MS and quantified using peak areas detected at 520 nm and based on external calibration via the reference substance cyanidin-3-O-glucoside (Behrendt, Röder, Will, Mostafa, Gonzalez-dominguez, et al. 2022).

4.1.3. Sample collection, storage

All the information related to blood and urine sample collection and storage are detailed in publication 2 (Behrendt, Röder, Will, Mostafa, Gonzalez-dominguez, et al. 2022). Feces

samples were collected at home by the volunteers using a sterile feces catcher (Abbexa BV, Zoetermeer, Netherlands) and a sterile universal fecal containers (Sarstedt AG & Co KG, Nümbrecht, Germany) and the samples were kept in cold (4ºC) until the volunteers arrived at the lab. Then, samples were lyophilized and stored at -80°C until further analysis.

4.1.4. Extraction of plasma-isolated ACNs and their metabolites before and after the intervention periods

Extraction of plasma ACNs and their metabolites (PAMs) was done using solid phase extraction (SPE) method. In brief, 1 mL of plasma was acidified with 30 µL of 50% aqueous formic acid before being loaded onto an Oasis-HLB (1 mL/30 mg) SPE cartridge, then it had been preconditioned with 1 mL of methanol and 1% formic acid. After that, 1 mL of acidified water (1% formic acid) was added. After washing the cartridge with 1 mL of acidified water, PAMs were eluted with 1 mL of acidified methanol. Following that, the eluates were kept for dryness under N_2 for about 3h (Behrendt, Röder, Will, Mostafa, Gonzalez-dominguez, et al. 2022; Mostafa et al. 2023).

4.1.5. In-vitro studies: Cell culture of colon and pancratic cancer cells

Human colon carcinoma cell lines (HT-29 and Caco-2) and pancreatic carcinoma cell lines (PANC-1 and AsPC-1) were obtained from American Type Culture Collection (ATCC) and cultured according to ATCC guidelines (http://www.lgcstandards-atcc.org/). HT-29 is a cell line with epithelial morphology that was isolated in 1964 from a primary tumor obtained from a 44 year old female patient with colorectal cancer, while Caco-2 are epithelial cells isolated from colon tissue derived from a 72 year old male with colorectal adenocarcinoma. PANC-1 is an epithelioid carcinoma cell line derived from ductal cell origin of the human pancreas from a 56 year old Caucasian male, while AsPC-1, a human pancreatic adenocarcinoma cell line derived from metastatic sites (ascites) of a 62 year old Caucasian female. All cells were grown in in DMEM (Dulbecco's modified Eagle's medium) supplemented with 5 mM L-glutamine, 1mmol/L sodium pyruvate and 10% fetal calf serum (FCS) at 37°C in a humidified atmosphere containing 5% CO2. More details can be found in

publications 2 and 3 (Behrendt, Röder, Will, Mostafa, Gonzalez-Dominguez, et al. 2022; Mostafa et al. 2023).

4.1.6. Cancer cell migration assays

Tumor cell migration was assessed in a Boyden membrane chamber with the use of the CytoSelect 24-well Cell Migration Assay.

In colon cancer migration assay, the feeder chamber trays were coated with 100 µL of 10 µg/mL collagen and then aspirated until dryness. Cells at density of 1 X 10^5/mL were seeded in DMEM with 1% of fetal calf serum (FCS) and PAMs from participants (day 0 vs. day 28) and added to the upper side of the chamber, whereas DMEM with 10% of FCS was added to the lower side of the chamber.

In pancreatic cancer migration assay, the chamber trays were coated with 50 µL of 0.1% fibronectin and then aspirated until dryness. After that, Human umbilical vein endothelial cells (HUVECs) which were obtained from pooled donors (from four different umbilical cords) were seeded onto the fibronectin-coated inserts and allow to grow confluent. Transepithelial electrical resistance (TEER) was determined before the experiments. A TEER value $\geq 250\ \Omega$ per cm^2 was used as an indicator for an intact endothelial layer suitable to be used for functional studies. Pancreatic cancer cells were seeded in Endothelial growth medium II supplemented with 2.5% FCS containing diluted PAMs from participants (day 0 vs. day 28), whereas Endothelial growth medium II supplemented with 12.5% FCS was added to the lower basal chamber.

For both migration assays, the cells were incubated in the feeder tray for 36 h at 37°C, and cells on the lower side were then detached from the membrane using a cell detachment solution and afterwards lysed with fluorescent dye-containing buffer. The extent of migration was assessed by the intensity of the fluorescence signal with a microplate fluorescence reader. The number of migrated cells was determined according to a calibration curve (500-15000 colon cancer cells and 0–7500 pancreatic cancer cells). More detailed can be found in publications 2 and 3 (Behrendt, Röder, Will, Mostafa, Gonzalez-Dominguez, et al. 2022; Mostafa et al. 2023).

4.1.7. Analysis of CAMs, NF-kB p65 detection and FAK activation in pancreatic cancer cell migration

Basal expression of adhesion molecules and VEGF-R (Vascular Endothelial Growth Factor-receptor) were analyzed under pre-confluent (PANC-1 and AsPC-1) or post-confluent conditions (HUVECs). Pancreatic cancer cells and endothelial cells were washed twice with phosphate buffered saline (PBS) solution and detached with TrypLE™ Express solution for cancer cells or Accutase solution (0.15 ml/cm^2) for endothelial cells. After detachment cells were centrifugated at 220 g for 3 min at RT. Supernatant was decanted and cell pellet was resuspended in cold 1000 µL MACS Running Buffer (pH 7.2) (Miltenyi, Bergisch Gladbach, Germany). 100 µl cells suspension was measured automatically by flow cytometry with MACS Quant 10 (MQ10) and quantification was performed using the MACSQuantify Version 2.13.2 software (Miltenyi, Bergisch-Gladbach, Germany) by comparing the mean fluorescence intensities (MFI). Commercially available enzyme linked immunosorbent assays (ELISA) for FAK and NF-kB were used to determine phosphorylated levels of FAK at tyrosine residue 397(FAK [pY397]) in prepared cell lysates after incubation and the InstantOne™ ELISA for measurement of phosphorylated human NF-kB p65 in cell lysates. Cells were lysed using the recommended cell extraction buffer after incubation with PAM (day 0 vs. day 28) and lysates were collected and stored at −80 °C. Optical density was measured at 450 nm and pFAK and pNF-kB protein expression was calculated using a four-parameter algorithm. The results are expressed as means with standard deviation (mean ± SD). More details can be found in publication 3 (Mostafa et al. 2023).

4.1.8. Rapid Folin-Ciocalteu (F-C) assay: Urine total polyphenol quantification

F-C assay was done to determine the total phenolic content (TPC) in urine, which is being used as an indicator for the total polyphenol intake, bioavailability and total phenolic accumulation in urine (Medina-remón et al. 2009; Cherubini et al. 2015). In a thermo microtiter 96-well plate, 170 µL of Milli-Q water, 15 µL of urine extract, 12 µL of the Folin-Ciocalteu reagent, and 30 µL of 20% sodium carbonate were mixed. The absorbance at 750 nm was measured using a UV/VIS Thermo Multiskan Spectrum spectrophotometer after 1h of incubation at room temperature in the dark. Gallic acid was used as a standard and

concentrations in the samples quantified as gallic acid equivalents (mg/L). All samples were processed in triplicate, with a <10% coefficient of variation (CV). The results were multiplied by the urine output and expressed in mg of gallic acid equivalents (GAE)/24 h. We used a 100 mg/l gallic solution as a standard and the calibration curve was made with 5 concentrations at 0, 1, 2, 4 and 8mg/l to check the linearity. The absorbances were then plotted against the standard concentrations. Mean absorbance and %RSD of the 3 replicates of each sample were calculated.

4.1.9. Sample preparation for targeted metabolomics analyses

For the preparation of plasma samples for metabolomics analyses, a protein precipitation method was used. Because of the poor stability of ACNs during sample preparation, this method was created and modified based on previous literature to be suitable with ACNs quantification as it showed higher recovery for ACNs metabolites than other methods (Liu and Michel 2018). The precipitation solution was composed of cold acetonitrile that included 10 mM ammonium formate and 1.5 M formic acid. The reconstitution solution was water:acetonitrile (80:20, v/v) containing 0.1% formic acid and 100 µg/L of a mixture of internal standards (ISs). 100 µl of plasma and 400 µl of the protein precipitation solution were added to a Sirocco protein precipitation plate. The plate was then placed in a -20°C freezer for 10 minutes to aid in the precipitation of the protein. The extracts were applied to nitrogen positive pressure, collected in a 96-well collection plate, and then moved to a speed vacuum concentrator for dryness. Finally, the extract was transferred to Agilent well plates after being reconstituted with 100 µl of the reconstitution solution and the ISs mix for further analysis. (González-Domínguez et al. 2020). Figure 4.2 shows the workflow for plasma metabolomics experiments conducted in this thesis.

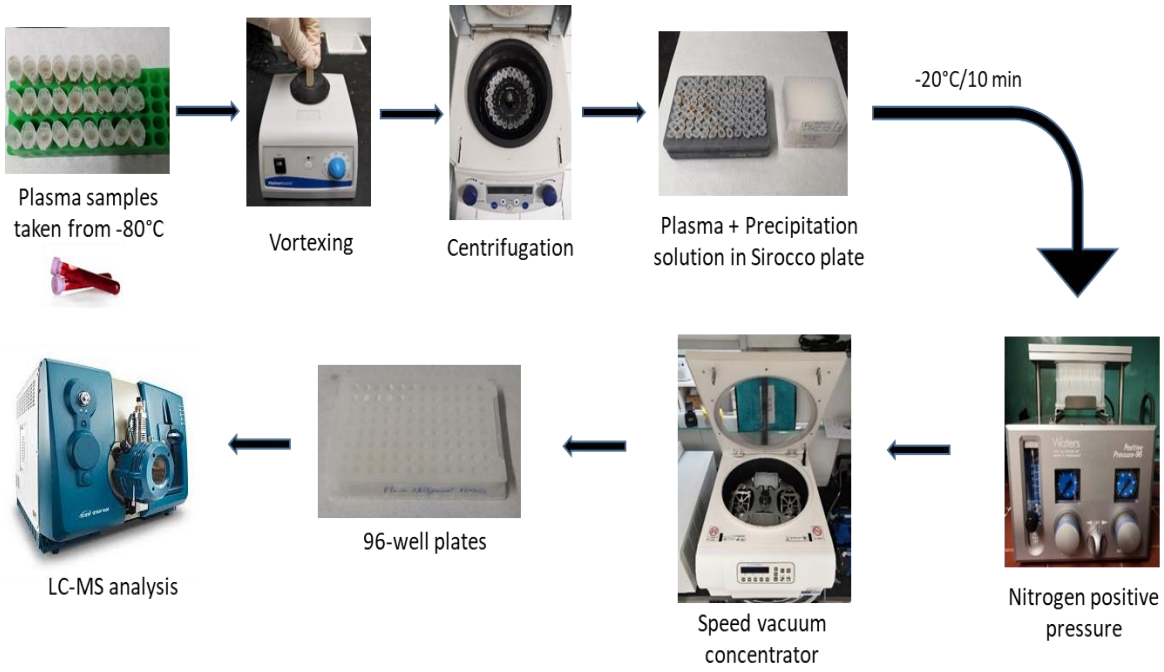


Figure 4.2: Plasma targeted metabolomics analysis conducted in this thesis

Two different procedures were used to prepare urine samples. The first treatment was the dilution method which is used for a wide range of metabolites, including small organic acids, amino acids, sugars, and other low molecular weight compounds. Urine samples were taken from a -80°C freezer and vortexed to be thawed before being centrifuged for 10 minutes at 4°C and 10,000g. The same ISs mixture mentioned previously was used. A 50 µl aliquot of the centrifuged urine was diluted with 200 µl of ultrapure water containing 0.1% formic acid (1:4 v/v) and the ISs solution before being transferred to 96-well plates for additional analysis. The second treatment was reversed SPE. SPE is consider as the gold standard technique for the cleanup of complex biological samples as it minimizes the interaction of the proteins and it is useful for the analysis of target metabolites and especially the extraction of polyphenols (Ajila et al. 2011). Using Oasis HLB plates, the plates were conditioned by adding 1 ml of methanol, and then 1 ml of water containing 1.5 M formic acid and 10 mM ammonium formate was added as the equilibration solution. After loading 1 ml of urine samples containing 20 µl of 2% orthophosphoric acid and the ISs mix, the equilibration solution was added again. Finally, 1.5 ml of the elution solution; a mixture of methanol containing 1.5 M formic acid and 10 mM ammonium formate was added. The extracts were then dried using a speed vacuum concentrator (Gyrozen®, scanspeed 32), reconstituted using

100 µl of the reconstitution solution and transferred to Agilent well plates for additional analysis. (González-Domínguez et al. 2020). Figure 4.3 is showing urine targeted metabolomics experiments conducted in this thesis.

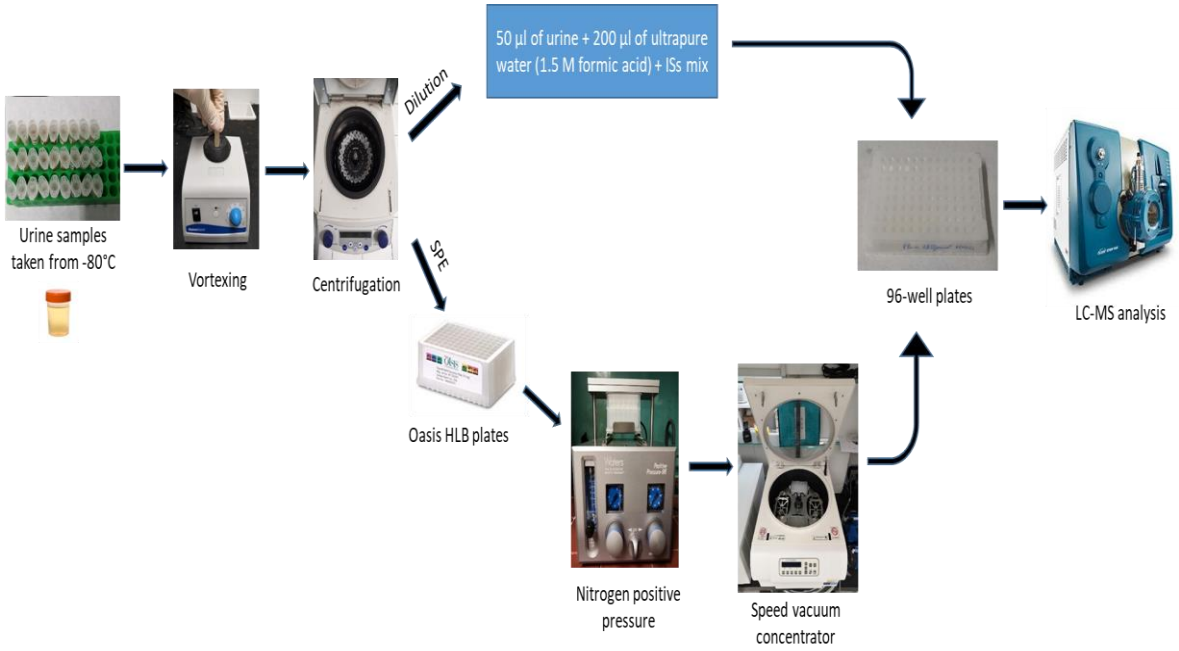


Figure 4.3: Urine targeted metabolomics analysis conducted in this thesis

For feces, the extraction solvent was an aqueous acidic solution (pH=3.1) containing 5 ml of Milli-Q water and 15 µl of concentrated acetic acid, and then mixed with 5 ml acetonitrile in a ratio of (1:1, v/v) (Karu et al. 2018; Deda et al. 2017). Lyophilized fecal samples were taken from -80ºC freezer and kept to be thawed at 6ºC. The extraction solvent was then added to 25 mg of the feces in a ratio of (1/2, W_f/V_s) and centrifuged for 30 min at 4ºC at 20,000g. The supernatant was then collected and filtered with syringe filters PTFE 0.22 µm. The filtered supernatant was then evaporated with speed vacuum concentrator to dryness. Finally, the dry feces extracted was reconstituted with a solution of water:acetonitrile (80:20 v/v) containing 0.1% formic acid (v/v) and 100 µg/l of IS mixture. (Karu et al. 2018; Deda et al. 2017).

4.1.10. UPLC-MS/MS targeted metabolomics analysis

The analysis of plasma, urine and fecal samples was performed on 1290 Infinity UPLC system (Agilent, USA) coupled to a QTRAP 6500 mass spectrometer equipped with Ion Drive Turbo V ion source (Sciex, USA) as shown in Figure 4.3. Luna Omega Polar C18 column, 100 mm x 2.1 mm (i.d. 1.6 um) with a porous polar C18 security guard cartridge (Phenomenex, USA) were used to perform the chromatographic separation. Mobile phases were based on two detection ion modes and were performed in separated runs in scheduled multiple reaction monitoring (sMRM); Positive ion mode consisted of (a) water and (b) acetonitrile and both of them were containing 0.5% formic acid, with a gradient program of 0−5 min 5%−50% (b); 5−8 min 50%−100% (b); 8−10 min 100% (b); 10−10.1 min 100%−5% (b); 10.1−12 min 5% (b). The negative ion mode consisted of (a) water containing 0.1% formic acid and 10 mM ammonium formate and (b) acetonitrile, with a gradient program of 0−8 min 5%−20% (b); 8−10 min 20%−100% (b); 10−12 min 100% (b); 12−12.1 min 100%−5% (b); 12.1−14 min 5% (b). Analytical validation was optimized by assessing the linearity of the calibration curves at 12 different concentration levels between 0.1-10000 μg/l. Quality controls, composed by a pool of samples of the study, were processed following the same procedures every 20 samples. Analyst 1.6.2 and Sciex OS software by Sciex were used for data acquisition and data processing, respectively. Finally, the coefficients of variation for areas, retention times and peak widths of the internal standards added to samples were calculated for analytical reproducibility assessment.

4.3. Observational Study. Diet, Cancer, and Health-Next Generations (DCH-NG) MAX study- (JPI-HDHL INTIMIC call, DiGuMet project, PCIN 2017-076).

4.2.1. Study design

The DCH-NG MAX study was a validation subsample within the Diet, Cancer, and Health-Next Generations (DCH-NG) cohort. The DCH-NG was an extension of the previous Diet, Cancer, and Health cohort (DCH) (Tjønneland et al. 2007). The DCH-NG included 39,554 participants from the DCH cohort, including biological children, spouses, and grandchildren (Petersen et al. 2022). Between August 2017 and January 2019, the DCH-NG MAX study recruited 720 volunteers with a Copenhagen residency who were 18 years old or older. The MAX study's main objectives were to validate a semi-quantitative food frequency questionnaire against 24-hour dietary recalls (24-HDR) and to look at the reproducibility of plasma and urine metabolomes as well as gut microbial stability over time. At baseline, 6, and 12 months, biological samples, health examinations such as anthropometric and blood pressure measurements, and two questionnaires about lifestyle and dietary habits were collected.

4.3.2. Dietary data

The 24-hour dietary recalls (24-HDR) were recorded at baseline, 6 and 12 months using a Danish version of Leeds University's web-based tool myfood24 (www.myfood24.org/) (Wark et al. 2018), which contained nearly 1,600 Danish food items. Participants reported all foods consumed the day before the examinations in either grams or standard portion size. The percentage of calories consumed using the energy equivalents for carbohydrates, proteins, and fat was used to calculate macronutrient intake. Recipes or dishes were assigned as complex food products. The McCance and Widdowson's Food Composition Table (Finglas et al. 2015), or recipes from the food frequency questionnaires in the DCH were used to have the standardized recipes (Tjønneland et al. 1991).

4.3.3. Dietary intake of ACNs and classification of dietary sources of ACNs in food groups

A protocol developed by the University of Barcelona, the Bellvitge Biomedical Research Institute (IDIBELL), and the Centro de Investigación Biomédica en Red (CIBER) was used to estimate the intake of polyphenols from 24-HDRs. A link between all 24-HDR food items or ingredients and the foods from the Phenol-Explorer database was created (Knaze et al. 2018). Individual (poly)phenol intake was measured in mg/day, and ACN intake from different foods was estimated using the sum of 71 individual ACNs from the Phenol-Explorer database. The DCH-NG MAX study's estimated intake of dietary (poly)phenols has previously been described (Lanuza, Zamora-Ros, Rostgaard-Hansen, Halkjær, et al. 2022). The total dietary ACN intake was estimated using 147 food items containing ACNs.

Dietary ACN intake was classified and added up by the following food groups: berries, foods containing at least 50% berries in their composition or recipe, dairy products with berries (including ice cream and yogurt), other fruits (including plums, cherries, apples, etc), non-alcoholic drinks (including fruit smoothies and juices), wines, vegetables, mixed dishes (meat or fish dishes with ACN-containing vegetables), and bakery (including pastry, biscuits, desserts, and waffles with berries or other ACN-containing preparations). Foods that did not contain ACN were not considered.

4.3.4. Sample collection, storage and preparation for analysis

Participants were instructed to fast for 1-9 hours (mean fasting time: 5 hours) on all examination days. At baseline time 0 (n = 624), 6 months (n = 380), and 12 months (n = 349), blood samples were collected in Vacutainer tubes containing lithium heparin. Plasma was obtained by centrifugation within 2 hours of blood extraction, and samples were stored at -80°C. The plasma samples were then delivered to the Danish National Biobank (DNB), aliquoted and, ultimately, shipped to the University of Barcelona, where they were stored at -80°C until metabolomics analyses. Other clinical laboratory parameters, such as haemoglobin A1c (HbA1c), serum lipids, and high sensitivity C reactive protein (hsCRP), were measured as previously described (17). Repeated measures of the plasma metabolome

at all three time points were used for metabolomics analysis. All samples were prepared and analyzed using the previously described targeted UPLC-MS/MS method.

4.4. Data analysis and statistics

4.4.1 Quality control and data pre-processing

To perform the quality control and pre-processing of metabolomics data the R/Bioconductor package 'POMA' developed in the group was used (https://github.com/nutrimetabolomics/POMAShiny) (Castellano-Escuder et al. 2021). First, metabolites with more than 40% missing values and those with a coefficient of variation (CV) > 30% in internal quality controls were removed. Then, the remaining missing values were imputed using the K-nearest neighbour (KNN) algorithm, and batch-effect correction was done using the ComBat function ('sva' R package) (Leek et al. 2012). Afterwards, data was normalized using auto-scaling. Moreover, distances to the group centroid were computed based on Euclidean distances to remove outliers from the data matrix [±1.5 × Interquartile range (IQR)].

4.4.2 Data analysis

In publication 2, linear mixed models (LMM) including treatment (juice/placebo), age, and sex as fixed effects and subject as random effect were used. *P*-values were adjusted for multiple comparisons using the Benjamini-Hochberg false discovery rate (FDR) and results were integrated in a volcano plot. Those metabolites with a log2FC > |0.584| (equivalent to a post-/pre-treatment ratio of 1.5) and an FDR-adjusted *p-value* <0.05 were selected as significant. Associations between plasma metabolites concentrations and migration or adhesion molecules experiments were tested using linear mixed adjusted for sex, age and treatment and participant as random effect.

In publication 3, the associations between consumption of total ACN and metabolome biomarkers were analyzed using the 'censReg' and 'plm' R-packages, (Croissant and Millo 2008). Due to the right-skewed distribution of the total ACN intake and the high percentage of zero values (24% of non-consumers ACNs), censored regression models were used. The models included age, sex, and BMI as covariates. The Benjamini-Hochberg technique was

used to adjust *p-values* for multiple comparisons, and an adjusted *p-value* of 0.05 was evaluated as statistically significant.

Mixed Graphical Models (MGM) with the 'mgm' R-package was used to conduct multivariate analyses in publication 3 (Haslbeck and Waldorp 2020). MGM are undirected probabilistic graphical models, where each node corresponds to one variable, and the edges between two nodes represent a conditional dependency adjusted for all other variables in the model. MGM specifications were set to allow the maximum number of interactions in the network. Variables in the model were dietary ACNs intakes by food categories (8 classes), and the whole metabolomic set of variables. There was a low agreement between repeated measurements for dietary ACNs intake from the different food sources for each individual (intra-class correlation coefficient <0.15). Therefore, all the observations of the study were considered independent and were included in the analysis. For visual clarity, only the first order neighbourhood was plotted.

Associations between metabolites and cardiometabolic risk factors were analyzed using LMM in a random intercepts model adjusted for age, sex and BMI. Standardized coefficients were plotted in a heatmap built using the 'pheatmap' R-package (Kolde R (2019). pheatmap: Pretty Heatmaps). *P*-values were adjusted for multiple comparisons using the Benjamini-Hochberg FDR. An FDR-adjusted *p*-value <0.05 was considered significant.

All statistical analyses were performed using IBM SPSS Statistics 25 (IBM, USA) and R version 4.2.1 (R foundation, Austria).

Figure 4.4 shows the metabolomics pipeline of this PhD thesis.

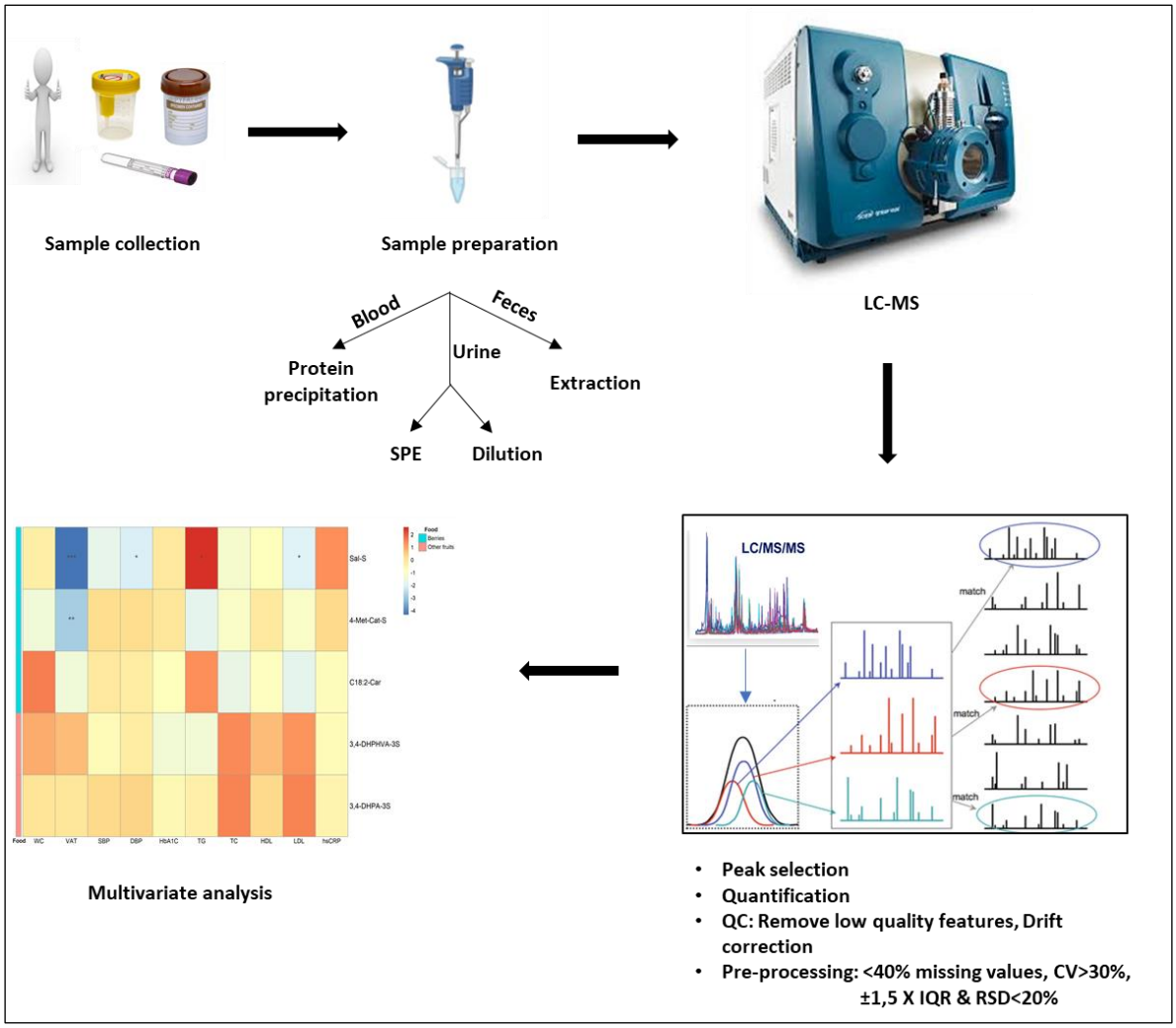


Figure 4.4: Targeted metabolomics workflow conducted in this PhD thesis

RESULTS

5. RESULTS

In this section, the work carried out throughout the thesis will be presented. All the articles are published in journals of first quartile in their research areas (Antioxidants, Biomedicine & Pharmacotherapy and Nutrients). The systematic review was sent for publications (under review). Before each publication, a summary of the objectives, results and the conclusion are briefly detailed in English and Spanish. The last section of this chapter (section 5.5) contains the work that hasn't yet been published.

5.1. Publication 1: Biomarkers of berry intake: Systematic review update

Manuscript sent for publication on 22nd February 2023. (Under review).

Abstract:

Berries are rich in polyphenols and these compounds may be beneficial to health. Estimating berry consumption through self-reported questionnaires has been challenging due to compliance issues and lack of precision. Estimation via food-derived biomarkers in biofluids has been proposed as a complimentary alternative. We aimed to review and update the existing evidence on biomarkers of intake for six different types of berries. A systematic literature search was performed on PubMed, Web of Science and Scopus from January 2020 until December 2022. Out of 42 papers, only 18 studies were eligible. Multi-metabolites panels are suggested for blueberry and cranberry intake, including hippuric acid with malvidin glycosides, and the glycosides of peonidin and cyanidin alongside the sulfate and glucuronides conjugates of phenyl-γ-valerolactone, respectively. No new metabolite candidates have been found for raspberries, strawberries, blackcurrants and blackberries. Further studies are encouraged to validate these multi-metabolite panels for improving the estimation of berry intake.

Resumen:

Las bayas son frutas ricas en polifenoles y estos compuestos pueden ser beneficiosos para la salud. Estimar el consumo de bayas a través de cuestionarios y encuestas nutricionales resulta difícil debido a problemas de cumplimiento y falta de precisión. Se ha propuesto que la estimación a través de biomarcadores alimentarios podría ser una alternativa complementaria. Nuestro objetivo fue revisar y actualizar la evidencia existente sobre biomarcadores de ingesta para seis tipos diferentes de bayas. Se realizó una búsqueda sistemática en PubMed, Web of Science y Scopus desde enero de 2020 hasta diciembre de 2022. De los 42 artículos, solo 18 estudios fueron elegibles. Se sugieren paneles de multi-metabolitos para la ingesta de arándanos y arándanos rojos, que incluyen ácido hipúrico con glicósidos de malvidina y los glicósidos de peonidina y cianidina junto con los conjugados de sulfato y glucurónidos de fenil-γ-valerolactona, respectivamente. No se han encontrado nuevos biomarcadores candidatos para frambuesas, fresas, grosellas negras y moras. En conclusión, nuevos estudios son necesarios para validar estos paneles de múltiples metabolitos para mejorar la estimación del consumo de bayas.

Biomarkers of berry intake: Systematic review update

Journal:	*Journal of Agricultural and Food Chemistry*
Manuscript ID	Draft
Manuscript Type:	Review
Date Submitted by the Author:	n/a
Complete List of Authors:	Mostafa, Hamza; Universitat de Barcelona Facultat de Farmacia i Ciencies de l'Alimentacio, Biomarkers and Nutrimetabolomics Laboratory, Department of Nutrition, Food Sciences and Gastronomy, Food Innovation Network (XIA), Nutrition and Food Safety Research Institute (INSA); Instituto de Salud Carlos III, Centro de Investigación Biomédica en Red de Fragilidad y Envejecimiento Saludable (CIBERFES) Cheok, Alex; King's College London Faculty of Life Sciences and Medicine, Department of Nutritional Sciences, School of Life Course and Population Sciences Meroño, Tomás; Universitat de Barcelona Facultat de Farmacia i Ciencies de l'Alimentacio, Biomarkers and Nutrimetabolomics Laboratory, Department of Nutrition, Food Sciences and Gastronomy, Food Innovation Network (XIA), Nutrition and Food Safety Research Institute (INSA); Instituto de Salud Carlos III, Centro de Investigación Biomédica en Red de Fragilidad y Envejecimiento Saludable (CIBERFES) Andres-Lacueva, Cristina; Universitat de Barcelona, Nutrition, Food Science & Gastronomy Rodriguez-Mateos, Ana; King's College London, Nutrition

Title: Biomarkers of berry intake: Systematic review update

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Results

Abstract

Berries are rich in polyphenols and these compounds may be beneficial to health. Estimating berry consumption through self-reported questionnaires has been challenging due to compliance issues and lack of precision. Estimation via food-derived biomarkers in biofluids has been proposed as a complimentary alternative. We aimed to review and update the existing evidence on biomarkers of intake for six different types of berries. A systematic literature search was performed on PubMed, Web of Science and Scopus from January 2020 until December 2022. Out of 42 papers, only 18 studies were eligible. The multi-metabolites panel is suggested for blueberry and cranberry intake, including hippuric acid with malvidin glycosides, and the glycosides of peonidin and cyanidin alongside the sulfate and glucuronides conjugates of phenyl-γ-valerolactone, respectively. No new metabolite candidates have been found for raspberries, strawberries, blackcurrants and blackberries. Further studies are encouraged to validate these multi-metabolite panels for improving the estimation of berry intake.

Keywords: Berries, Blueberry, Cranberry, Raspberry, Strawberry, Blackberry, Blackcurrant, BFIs

1. Introduction

Berries are fruits often included in healthy dietary patterns as they are rich in phytochemicals, fibre and micronutrients (1,2). Berries may play an important role in the prevention and treatment of chronic diseases such as cardiovascular diseases (CVD), cancer, diabetes mellitus (DM) and age-related cognitive decline, according to an increasing number of randomized controlled trials (RCTs) and epidemiological studies (3–5). However, to understand the relationship between berries and health, it is essential to be able to quantify their consumption accurately. The development of novel dietary assessment methods to estimate food consumption is a current focus in nutrition research. In particular, recent studies aim to discover and validate biomarkers of food intake (BFIs) in biofluids, typically in blood and urine. BFIs can be effectively used to: i) validate dietary intake questionnaires, ii) study physiological and pathological responses to food and food components, iii) provide information on inter-individual variability in food responses, and iv) help to formulate personalized dietary recommendations (6–8). However, only a very limited number of biomarkers for specific foods have been proposed. Currently, there are no validated biomarkers for berries.

Berries phytochemicals include anthocyanins, ellagitannins, flavonols, flavan-3-ols and phenolic acids (9). A large number of circulating metabolites, including phase II and gut microbial metabolites derived from these compounds have been reported after the consumption of different types of berries in human intervention studies (10–12). Previous studies have proposed ellagitannins and their gut microbial metabolites, urolithins (Uros) as biomarkers of strawberry and raspberry intake (13,14), aromatic compounds like furaneol as biomarkers of strawberry intake (15), and anthocyanins (ACN) like malvidin and its derivatives as biomarkers of blueberry intake (16,17). However, these metabolites are ubiquitous and can be found in many types of foods, such as nuts, grapes, or red wine, therefore they cannot be used as single biomarkers due to the lack of specificity coupled with their abundance in the habitual diet. Similarly, phenolic acids may be produced by gut microbial metabolism of berries phytochemicals, but they are also present in the daily diet in foods such as coffee, tea, bread, and other fruits and vegetables. A potential solution to this is to use a multi-biomarker panel as biomarker of intake, which has been proposed previously to be more advantageous over a single biomarker (18). In the last few years, a number of studies have provided additional information on the berry metabolome, therefore we aimed to update a previous investigation of biomarkers of berry intake (19) to capture additional candidates to improve the proposed metabolite panels.

2. Methodology

We conducted the current systematic review according to the PRISMA statement (Preferred Reporting Items for Systematic Reviews and Meta-Analysis) as detailed in Supplementary Table 1. Our review employed an extensive search using the following keywords (Fruit name* OR botanical name), AND (urine or plasma or serum or excretion or blood) AND (human* OR men OR women OR patient* OR volunteer* OR participant*) AND (biomarker* OR marker* OR metabolite* OR biokinetics OR biotransformation OR pharmacokinetics OR bioavailability OR ADME) AND (intake OR meal OR diet OR ingestion OR administration OR consumption OR eating OR drink) across three electronic databases: PubMed, Web of Science and Scopus. The previous search has covered published literature until December 2019. To continue upon that, our systematic search included trials which were conducted only on berries published, in English only, between January 2020 to December 2022. Short- and long-term human interventions studies conducted on berries intake were selected for this review. The main exclusion criteria were animal and *in-vitro* studies and papers assessed irrelevant diets as detailed in Figure 1.

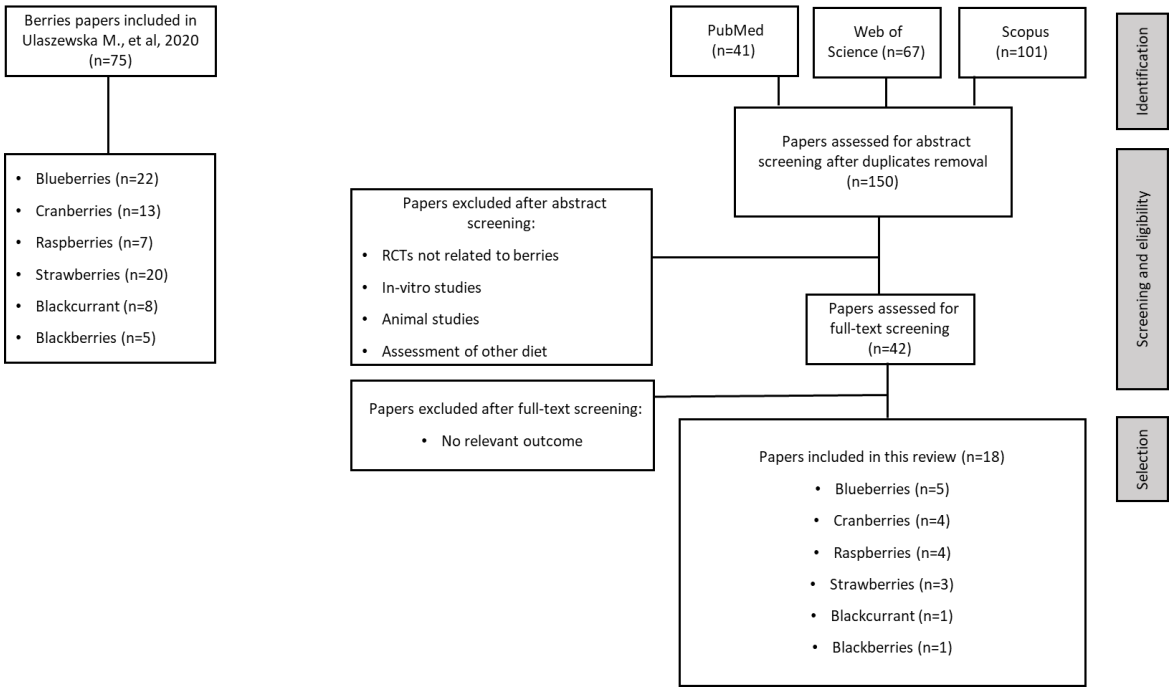
3. Results and discussion

We found 41 papers in PubMed, 67 papers in Web of Science and 101 papers in Scopus. Duplicated papers or papers found irrelevant to our objective were excluded from the review. After the inclusion and exclusion criteria, we obtained 42 papers that fulfilled the criteria for this review. After full-text screening, 18 papers were selected and analyzedas detailed in Figure 1 and Table 1.

3.1 Description of the selected trials

Among the selected 18 studies, 8 studies had a berry drink as an intervention (in the form of juices, nectars or frozen juices), two studies used extract capsules, one study used frozen berry and 7 studies used freeze-dried powder. Twelve studies included only healthy participants. The age of the participants in all the studies included in this review ranged from 21 to 75 years, except for one study, which was conducted on children (7-10 years old). Two studies had female participants only, while five studies had only male participants. There were 5 studies classified as acute interventions (single dose) while the long-term interventions were between 3 days and 90 days as shown in Table 1.

Results



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121 Figure 1: Study selection bibliography search flow diagram

122

Results

123 Table 1: List of the studies included for the assessment of biomarkers of berry intake

Reference	Intervention	Study characteristics	Analytical method	Candidate biomarkers (blood)	Candidate biomarkers (urine)
Blueberries (BB)					
(20)	50g freeze-dried powder No phytochemical intake reported	Double-blind parallel RCT Duration: 8 w 49 participants - At risk of MS - 55% females - Age: 22-53 y MS: metabolic syndrome	Targeted metabolomics LC-MS/MS analysis Plasma samples (w0 and w8) 6 identified metabolites	Increased - HA Decreased - Ornithine - Hypoxanthine - Diacylglycerol - Indoxyl *sulfate* - Ceramide	
(21)	26g freeze-dried powder Contains 1243mg of total pp	Double-blind parallel RCT Duration: Single dose 45 participants - With MS - 64% males - Age: 63.4 ± 7.4 y	UPLC-MS/MS analysis Serum samples (30, 60, 90, 120, 180, 360min and 24h), Urine samples (24h) 21 identified metabolites	- 2,6-dimeO-phenol - 3-(4-OH-3- meO-ph)-PrA - 3-OH-4-meO-PA - 3-meO-PA-4-*S* - 4-OH-HA - HA - HA-*S* - OH-meO-BA-*S* - 3-(3-meO-ph)-PrA - Methoxy-PA-gluc	- Phloroglucinaldehyde - 3-(3,4-diOH-ph)-PrA - 3-OH-HA - Syringic acid - 4-OH-BA - Isovanillic acid-gluc - Benzoylglutamic acid - 3-Caffeoylquinic acid - HA - BA-4-S - 3,4-OH-BA-3/4-*S* - DOPAC
(22)	24g freeze-dried powder Contains 36mg/g of total pp	Double-blind parallel RCT Duration: 90d 38 participants - Healthy - 68% females - Age: 60-75 y	UPLC-QQQ-MS analysis Plasma samples (0, 2h) (d0, d45, d90) 11 identified metabolites	- HA - Phloroglucinaldehyde - Syringic acid - Ferulic acid-gluc - Cya-3-galac - Cya-3--glu - Mal-3-galac - Mal-3-glu - Peo-3-xyl - Peo-3-gluc - Pet-3-glu	
(23)	25g of (Vaccinium Myrtillus (VM)) 25g of (Vaccinium Corymbosum (VC))	Single-blind parallel randomized trial Duration: Single dose	Untargeted metabolomics LC-HRMS analysis	After VM consumption: - Uric acid - Inosine - α-Hydroxyhippuric acid - Catechol-*S*	

Results

	Contains 39mg of catechin equivalent/g (total pp ~ 1g)	20 participants - Healthy - 1 male, 9 females - Age: 25-60 y	Serum samples (30, 60, 120, 240, and 360min) 12 identified metabolites	- me-catechol-*S* - Abscisic acid glucuronide - Azelaic acid - OH-ph-PrA-*S* After VC consumption: - Caprylic acid (hydroxyl octanone) - Tetrahydro-me-ß-carboline dicarboxylic - Octahydro-methyl-ß-carboline dicarboxylic - Citric acid for both VM and VC.	
(24)	13.3g BB drink Contains 766mg of total pp	Single-blind between-groups trial Duration: 4w 15 participants - Healthy - 7 males, 8 females - Age: 7-10 y	UHPLC-Q-TOF-MS analysis Urine samples (24h) 2 identified metabolites		- HA - Dihydrocaffeic acid-3-*S*
Cranberries (CB)					
(10)	CB Juice Contains 375, 716, 1131, 1396, 1741mg of total flavan-3-ols	Double-blind six-arm crossover RCT Duration: Single dose 10 participants -Healthy - Males - Age: 18-35 y	Targeted UHPLC-ESI-QqQ-MS/MS analysis Plasma samples (0, 1, 2, 4, 6, 8 and 24h), Urine samples (0, <8, 8-24h) 22 identified metabolites	- 5-(diOH-ph)-γ-VL-gluc (3′,4′,5′) - 5-(5′- OH-ph)-γ-VL-3′-gluc - 5-(3′,5′-diOH-ph)-γ-VL - 5-(diOH-ph)-γ-VL-*S* (3′,4′,5′) - 5-ph-γ-VL-4′-gluc - 5-(3′-OH-ph)-γ-VL-4′-gluc - 5-ph-γ-VL-*S*-gluc isomer (3′,4′) - 5-(4′-OH-ph)-γ-VL-3′-gluc - 4-OH-5-(OH-ph)-VA-*S* (3′/4′) isomer 1 - 4-Hydroxy-5-(OH-ph)-VA-gluc (3′/4′) - 5-(5′-OH-ph)-γ-VL-3′-*S* - 5-ph-γ-VL-meO-gluc isomer (3′/4′) - 5-OH-ph -γ-VL-meO-gluc (3′,4′,5′) - 5-ph-γ-VL-3′-gluc - 4-OH-5-(OH-ph)-VA-*S* (3′/4′) isomer 2	- 5-(5′-OH-ph)-γ-VL-3′-gluc - 5-ph-γ-VL-4′-gluc - 5-(3′-OH-ph)-γ-VL-4′-gluc - 5-ph-γ-VL-*S*-gluc isomer (3′,4′) - 5-(4′-OH-ph)-γ-VL-3′-gluc - 5-(3′,4′-diOH-ph)-γ-VL - 5-(5′-OH-ph)-γ-VL-3′-*S* - 5-ph-γ-VL-meO-gluc isomer (3′/4′) - 5-ph-γ-VL-3′-gluc - 5-(OH-ph)-γ-VL-*S* (3′,4′ isomers) - 5-ph-γ-VL-meO-*S* (3′,4′) isomer 1 - 5-ph-γ-VL-3′-*S* - 5-ph-γ-VL-meO-*S* (3′,4′) isomer 2

Results

				- 5-(OH-ph)-γ-VL-meO-*S* (3′,4′,5′) - 5-(OH-ph)-γ-VL-*S* (3′,4′ isomers) - 5-ph-γ-VL-4′-*S* - 5-ph-γ-VL-meO-*S* (3′,4′) isomer 1 - 5-ph-γ-VL-3′-*S* - 5-ph-γ-VL-meO-*S* (3′,4′) isomer 2	
(25)	9g powder Contains 525mg of total pp	Double-blind parallel RCT Duration: 1 month 45 participants - Healthy - Males - Age: 25 ± 3 y	Targeted quantitative UPLC-MS analysis Plasma samples (0, 2h) (d1, 1 month), Urine samples (24h) (d1, 1 month) 130 identified metabolites	- 1 Flavonol - 2 Benzene diols and triols - 2 BAL - 5 HAs - 11 BAs - 12 CAs - 6 PAs - 12 ph-PrAs - 5 ph-γ-VLs and ph-VAs Increased after 2h: 13 in plasma Increased after 1 month: 4 in plasma	- 5 Flavonols - 7 Benzene diols and triols - 3 BAL - 5 HAs - 14 BAs - 15 CAs - 6 PAs - 11 ph-PrAs - 8 ph-γ-VLs and ph-VAs Increased after 1 day: 13 in urine Increased after 1 month: 13 in urine
(26)	6 bottles (250ml per bottle, twice a day) of 54% CB juice Contains 913±7mg of total pp Mean±SD	Crossover RCT Duration: 3d 15 participants -Healthy - Females - Age: 21-29 y	Untargeted UHPLC-Q-orbitrap-HRMS-based metabolomics analysis Spot urine samples (d0, after d3) 16 identified metabolites		Exogenous metabolites: - Quinic acid - Coumaric acid - 4-OH-5-(OH-ph)-VA-*S* - 5-(diOH-ph)-γ-VL-*S* - Diphenol-gluc - 3,4-diOh-ph-PA - 3-(OH-ph)-PA - 4-me-gallic acid, - triOH-BA - 1,3,5-trimeO-benzene Endogenous metabolites: - Homocitric acid - HA - 3-OH-3-carboxy-me-adipic acid - (2)3-isopropylmalate - Pimelic acid - N-acetyl-l-glutamate 5-semialdehyde
(27)	6 bottles (250ml per bottle, twice a day) of 54% CB juice	Double-blind crossover RCT	UPLC-MS untargeted metabolomics analysis	Exogenous metabolites: - Quinic acid ǂ - 3-(OH-ph)-PA ǂ	

Results

	Contains 913±7mg of total pp Mean±SD	Duration: 21d 16 participants - Healthy - Females - Age: 21-29 y	Plasma samples (d0, d3, d21) 25 identified metabolites	- (*S*)-Homostachydrine - et-(methylthio)methyl disulfide - Catechol-*S* ǂ - Vanilloloside ǂ - *S*-Acetyl dihydroasparagusic acid - Pyrocatechol - Guaiacol Endogenous metabolites: - HA ǂ - Glycerol 3-phosphate - diOH-quinoline ǂ - OH-pyruvic acid - 3,4-diOH-ph-glycol - Guanidoacetic acid - 2-Chloromaleylacetate - 2-Phenylacetamide - Tyrosine ǂ - 3-Isopropylmalate - 2-Chloromaleylacetate - Lanthionine ketimine - Prolyl-Hydroxyproline - Tyramine-*O*-*S* - (3,4,5,6-tetrahydroxyoxan-2-yl)me-4-OH-benzoate - {4-[2,3-dioxo-3-(2,4,6-trihydroxy-3-meO-ph)propyl]-2-OH-6-meO-ph}oxidanesulfonic acid ǂ Identified in a previous study (28)	
Raspberries (RB)					
(29)	- 250g frozen RP Contains 203.6±11.1mg/meal of total pp Mean±SD - 125g frozen RP	Single-blind three-arm crossover RCT Duration: Single dose 32 participants - 12 preDM-IR and 11 healthy - 15 males, 17 females - Age: 34 ± 12	UHPLC-QTOF analysis Plasma samples (0, 0.5, 1, 2, 4, 6, 7, 8, and 24h) 24 identified metabolites	- Cya-3-sop - Cya-3-glu - Cyanidin glucosylrutinoside - Cya-3-rut - Pel-3-sop - Pel-3-glu - me-cya-sop - me-cya-3-glu - 8-OH-Uro-3-gluc - Uro-3-gluc - 4′-OH-CA	

Results

	Contains 101.8±5.6mg/meal of total pp Mean±SD			- OH-CA - CA-gluc - 4′-OH-3′-meO-CA - OH-meO-CA - meO-CA-gluc isomer 1 - etO-CA-gluc isomer 2 - 2,4,6-triOH-BAL - 3,4-diOH-BA - 2,3- diOH-BA - 4-OH-PA - diOH-ph-PA isomer - HA - HA-gluc	
(30)	Frozen RB drink Contains 388.4±3.3mg of total pp Mean±SD	Single-blind crossover RCT Duration: 4w 35 participants: - 25 preDM-IR and 10 healthy - 17 males, 18 females - Age: 34 ± 3	UHPLC-QQQ analysis Plasma samples (0, 0.5, 1, 2, 3, 4, 24h), Urine samples (0, 1,2,3,4, 24h) 123 identified (poly)phenolic metabolites	- 13 ACN derivatives - 7 Uros derivatives - 9 ph-γ-VLs derivatives - 94 Phenolic acid derivatives: - 10 BALs - 24 CAs - 19 ph-PrA - 17 PAs - 16 BAs - 8 HAs Increased: - Total Uros - Total ph-γ-VL - Select PA(CAs, ph-PrAs and HAs) Decreased: - Total ACN	Increased: - Total Uros - Total ph-γ-VL
(31)	280g frozen RP No phytochemical intake reported	Two-arm parallel RCT Duration: 8w 48 participants - Obese - 16 males, 32 females - Age: 18-60 y	Targeted metabolomics analysis using MxP® Quant 500 kit Plasma samples (w0, w4, w8) 10 identified metabolites	Exogenous metabolites: - Cholesterol 1-pentadecanoate - 1-Palmitoyl-2-palmitoyl-3-docosahexaenoyl-glycerol - 1-Octadecanoyl-2-(9Z-hexadecenoyl)-3-(9Z-tetradecenoyl)-glycerol - 1-Palmitoleoyl-2-palmitoleoyl-3-linoleoyl-glycerol - 1-Arachidonyl-2-docosapentaenoyl-sn-glycero-3-phosphocholine	

Results

				- 1-Octadecanoyl-2-octadecanoyl-3-(9Z-tetradecenoyl)-glycerol Endogenous metabolites: - β-Alanine - Trimethylamine N-oxide - Deoxycholic acid glycine conjugate - Hexosylceramide	
(32)	- RP nectar of 10g, 20g RP confection of 10g, 20g - Nectar: contains 17 and 46mg of ETs. Confection: contains 25 and 50mg ETs ETs: ellagitannins	Five-arm parallel RCT Duration: 4 weeks 40 participants - Healthy - Male - Age: 60.2 ± 7 y	UPLC/MS/MS analysis Plasma samples, Urine (spot and 24h) 5 identified metabolites	- Uro A - Uro C	- Uro A - Uro B - Uro C - Uro D - dime-ellagic acid
Strawberries (SB)					
(33)	50g freeze-dried powder Contains 450.7mg of total pp	Double-blind crossover RCT Duration: 4w 34 participants - Obese - 17 males, 17 females - Age: 52.6 ± 7.1 y	UPLC-QQQ analysis Plasma (w0, w4) 37 identified bile acids (BA) species	Decreased: - Secondary BAs - Deoxycholic acid - Lithocholic acid (and their glycine conjugates) - Glycoursodeoxycholic acid	
(34)	50g freeze-dried powder Contains 450.7mg of total pp	Double-blind crossover RCT Duration: 4w 34 participants - With MHC - 17 males, 17 females - Age: 53 ± 1 y	Targeted metabolomics UHPLC-ESI-MS/MS analysis Plasma samples (0, 1h) 17 identified phenolic metabolites	8 increased: - 3-meO-BA-4-S - 3-meO-PA - 3-OH-ph-*γ*-VL-4-*S* - 4-OH-PA - 4-OH-3-meO-BA-me ester - 3-(4-OH-ph)-PA-3-*S* - OH-CA-*O*-gluc - 4-OH-3,5-dimeO-PA 4 decreased:	

Results

				- 4-meO-CA - 3-me-HA - OH-BAL-*O*-gluc - 3-(4-meO-ph)-PA-3-gluc	
(35)	- 26g SB powder + Beige diet - Beige diet only No phytochemical intake reported	One-arm parallel trial Duration: 4w for (Beige diet + SB), 2w for Beige diet only 15 participants - Healthy - 6 males, 8 females - Age: 18-55 y	LCMS/MS analysis Serum samples (w0, w4, w6), Urine samples (24h) (w0, w4, w6) 4 identified metabolites	- Pel-3-gluc	- Pel-3-gluc - Uro A-gluc - dime-ellagic acid-gluc
Blackcurrants (BC)					
(36)	300mg extract capsule Contains 105mg of ACN	One-arm trial Duration: Single dose 20 participants -Healthy - 11 males, 9 females - Age: 28 ± 7 y	Reversed-phase HPLC analysis Plasma samples (0,1,1.5,2,3,4,5,6h) 2 identified metabolites	- Gallic acid - PCA	
Blackberries (BLB)					
(37)	400ml of commercial nectar Contains 100mg of Cya-3-glu	One-arm trial Duration: Single dose Two participants - Healthy - Age and gender: unreported.	LC-IMS-QTOF-MS and LC-LIT-MS analysis Urine samples (24h) 2 identified metabolites		- Two sulfated cya derivatives - One sulfated cya-3-gluc

BB: blueberries, CB: cranberries, RB: raspberries, SB, strawberries, BC: blackcurrants, BLB: blackberries, VM: vaccinium myrtillus, VC: vaccinium corymbosum, preDM-IR: prediabetes and insulin resistance, MHC: moderate hypercholesterolemia, HA: hippuric acid, 4-OH-BA: 4-Hydroxybenzoic acid, DOPAC: 3,4-Dihydroxyphenylacetic acid, BA: Benzoic acid, CA: cinnamic acid, 4-OH-BA: 4-Hydroxybenzoic acid, 3,4-OH-BA: 3,4-Hydroxybenzoic acid-3/4-*sulfates*, BAL: benzaldehydes, Cya-3-galac: cyanidin-3-galactosdie, Cya-3-glu:cyanidin-3-glucoside, Mal-3-galac: malvidin-3-galactoside, Mal-3-glu: malvidin-3-glucoside, Peo-3-xyl: peonidin-3-xyloside, Peo-3-gluc: peonidin-3-glucoronide, Pet-3-glu: petunidin-3-glucoside, Pel-3-gluc: pelargonidin-3- glucuronide, OH-ph: hydroxyphenyl, diOH-ph: Dihydroxyphenyl, ph: phenyl, VL: valerolactones, VA: valeric acid, PCA: protocatechuic acid, PrA: propionic acid, ph-PrA: phenylpropionic acid, PA: phenylacetic acid, 4-OH-PA: 4-hydroxyphenylacetic acid, me: methyl, dime: Dimethyl, meO: methoxy, dimeO: dimethoxy, meO-ph: methoxyphenyl, et: ethyl, etO: ethoxy, Uros: urolithins, Uro A: urolithin A, Uro B: urolithin B, Urol C: urolithin C, Uro D: urolithin D.

3.2 Biomarkers of berry intake

Blueberry

Twenty two studies investigated the metabolic fate of polyphenols after blueberry intake in Ulaszewska M. et al.'s systematic review (16,17,46–55,38,56,39–45). They concluded that the major ACN found in blood and urine were delphinidin and malvidin glycosides. Various phenolic metabolites were also found after the acute and chronic intake of blueberry such as benzoic, ferulic, catechol, hippuric and phenyl valeric acids, and phenyl-valerolactone derivatives. However, these metabolites were not specific to blueberry intake and can also be produced after the intake of many other fruits and vegetables. Thus, there were no metabolites identified as BFIs for blueberry and it was suggested that more trials were needed. In our systematic search, five additional studies that investigated plasma or urine metabolites after blueberry consumption were included (20–24). Two of them were acute, single-dose studies while the rest were long-term trials. One study administered a drink while four studies used reconstituted freeze-dried powder. Only one study detected ACN in plasma namely cyanidin-3-galactoside (cya-3-galac), cyanidin-3-glucoside (cya-3-glu), malvidin-3-galactoside (mal-3-galac), peonidin-3-xyloside (peo-3-xyl), peonidin-3-glucuronide (peo-3-gluc) and petunidin-3-glucoside (pet-3-glu) (22). Phenolic compounds produced via gut microbial metabolism of ACN were found in all the five studies. Among them, syringic acid, isovanillic acid, ferulic acid, benzoic acid, catechol and their derivatives, 3-caffeoylquinic acid, hydroxyphenylpropionic acid *sulfate* (OH-ph-PrA-*S*), 2,6-dimethoxyphenol, 3,4-dihydroxyphenylacetic acid (3,4-diOH-PA) and others are detailed in Table 1. Interestingly, hippuric acid (or hydroxyhippuric acid) was the only common phenolic compound found in all of the studies regardless of the type of biofluid, study design or trial duration. In Lapo Renai et al. (23), they identified 12 metabolites in serum in their untargeted metabolomics technique after a single dose of a vaccinium supplement, including some phenolic compounds, uric acid, inosine, and abscisic acid glucuronide (Table 1.). They also revealed that five from the 12 metabolites were not previously related to the exposure to blueberry. These were citric acid, azelaic acid, caprylic acid and two derivatives of ß-carboline dicarboxylic acid. However, they have concluded in their study, in agreement with previous data, that these metabolites are not specific to blueberry intake as they might be biomarkers of other fruits and vegetables consumption, as well as alcohol, coffee and fructose intake (57–60). Some endogenous metabolites were increased or decreased in blood samples after the intake of blueberry, nevertheless, endogenous metabolites could be affected with various factors inter-individually

which make it unrealistic to be used as specific biomarkers for blueberry intake (Table 1). In line with the result from the previous review, we can suggest that the presence of hippuric acid with malvidin glycosides could be an indicator of blueberry intake, and could be used potentially to assess compliance in human studies, however, it is not specific enough to be consider a biomarker of blueberry intake within the overall diet of an individual. In addition, due to the low anthocyanin levels in biofluids, and the fact that they are highly unstable compounds, intact anthocyanins are not an optimal biomarker of intake.

Cranberry

Thirteen studies using untargeted and targeted approaches in plasma and urine after cranberry consumption were included in Ulaszewska M. et al. systematic review (61,62,71–73,63–70), revealing that the most abundant ACN found were the glycosides of arabinoside, glucosides and galactosides of peonidine and cyanidin. In addition, various phenolic metabolites were identified after cranberry intake such as the sulfate conjugates of catechols, ferulic acid, valerolactones and coumaric alongside other metabolites such as hippuric acid, citramalic acid and the derivatives of terpenes and iridoids. Nevertheless, these metabolites are not specific BFIs for cranberry intake as they are also metabolites formed after the consumption of many other polyphenol-rich foods. In this work, four studies (10,25–27) explored the plasma and urine metabolome of cranberry intake. Two (10,25) have used targeted analysis; whereas the other two have used untargeted analysis (26,27). They are all RCTs with intervention ranging from a single dose (10) up to a daily intake of a month. Only one study administered cranberry powder dissolved in water (25), while the rest were all in the form of juices. As covered previously, Liu et al. (65) revealed through their untargeted plasma analysis several endogenous and exogenous metabolites discriminating the intake of cranberry juice vs. apple juice. Recently, the same group has expanded their investigation following a similar approach published in two separate studies (26,27). In addition to their previous attempt, newly identified exogenous discriminant metabolites include 4-hydroxy-5-(hydroxyphenyl)-valeric acid *sulfate*, 5-(dihydroxyphenyl)-γ-valerolactone *sulfate*, pyrocatechol, guaiacol, (S)-homostachydrine, ethyl (methylthio)methyl disulfide, and *S*-Acetyl dihydroasparagusic acid collectively. Though these compounds are not normally associated with cranberry exposure, they are largely common in many foods. Additional endogenous metabolites were also found (Table 1); however, they should not be given consideration as BFIs due to their susceptibility to changes by many other factors. Another study detected 56 and 74 (poly)phenol metabolites in plasma and urine respectively via a targeted approach (25) after participants consumed 9 g

of reconstituted cranberry powder daily for a month. Overall, 13 metabolites were found to have increased in plasma as well as 13 in urine samples. However, these are mainly derivatives of phenyl-γ-valerolactones (ph-γ-VLs), phenylvaleric acids (ph-VAs), cinnamic acids (CAs) and benzoic acids (BAs) which were previously excluded as BFIs. Another study from the same team (10) performed a targeted analysis of 22 ph-γ-VLs and ph-VAs in plasma and urine after an increasing dose (375 – 1741 mg) of total flavan-3-ols. The ph-γ-VLs in both plasma and urine showed a linear dose-response, with the glucuronide and sulfate derivatives of 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone being the most dominant metabolites. Although 5-(3′,4′-Dihydroxyphenyl)-γ-valerolactone and some of its derivatives were previously ruled out as BFIs in Ulaszewska M. et al., here the authors suggested that its *sulfate* and glucuronide conjugates could serve as a biomarker for the intake of cranberry flavan-3-ols in the context of controlled clinical trials, as they are not specific enough for being biomarkers of cranberry intake. These gut microbial metabolites have been proposed as biomarkers of flavan-3-ol intake in previous studies, and have been successfully used in an epidemiological study investigating associations between flavan-3-ol intake and blood pressure (74,75). Taken together, it is clear that these conjugates cannot be considered as specific biomarkers of cranberry intake, perhaps they could be used as indicators for intake when they appear alongside some ACN associated with cranberry intake, possibly as a collective multi-biomarker panels. Further studies are warranted for this.

Raspberry

Seven studies investigating circulating raspberry metabolites were considered in the previous systematic review (12,76–81). Various ACN, phenolic and ellagitannins metabolites were found in low concentrations in plasma and urine such as cya-3-glu, hippuric acid, ferulic acid, phenyl acetic acid, urolithins and ellagic acid derivatives but these metabolites are not specific to raspberry consumption. Some animal models detected the formation of raspberry ketone 4-(phenylhydroxyphenyl)-2-butanone and its derivatives 4-(4-hydroxyphenyl)butan-2-ol and 4-OH-PA in urine and they have been considered as BFIs for raspberries (80). These ketones can be rapidly absorbed from the gastrointestinal tract and are highly exclusive to raspberry. It is surprising that raspberry ketone metabolites, even until today, were only being studied in animal models but never in humans. Nevertheless, due to the widespread use of the raspberry ketone as a food additive, they are not suitable to be used as single biomarkers. The use of raspberry ketones along with other raspberry derived metabolites such as urolithins as a multi BFIs was proposed to be an indicator of raspberry intake. The authors concluded that more

clinical trials on human are still in need to validate these metabolites for use as BFIs of raspberries. Our systematic review captured four new publications looking at the metabolome after raspberry consumption (29–32). Two studies from the same research group (29,30) collectively measured up to 123 (poly)phenolic metabolites in plasma and urine of prediabetic individuals after single-dose and a month of continuous raspberry consumption, respectively. Their targeted analyses revealed that several metabolite groups including ACN, Uros, ph-γ-VLs and phenolic acids were altered across the biological fluids. Cya-3-sophoroside (cya-3-sop), cya-3-rutinoside (cya-3-rut) and cyanidin-3-glucoside (cya-3-glu) were found as the major ACN. Despite ACN being established as the most characteristic polyphenol in berries, they inherently suffer from low bioavailability. They are also structurally unstable and readily degrade into smaller phenolic metabolites making it problematic as BFIs. There are also no specific ACN for each individual berry and ACN are largely common across red and purple foods such as grapes, wine, apples, onions, aubergines or plums. One study (31) performed targeted metabolomics in obese participants after 8 weeks frozen raspberry consumption. They reported changes in 10 plasma metabolites (4 endogenous and 6 exogenous). The exogenous metabolites were made up of triacylglycerols, glycerophospholipids and cholesterol esters (Table 1). These compounds are omnipresent and can be found across a wide range of animal and plant-based products rendering them ineffective BFIs. Lastly, Roberts et al. (32) focused on metabolism of ellagitannins in male individuals upon 4 w consumption of raspberry products and quantified Uro A-D and dimethylellagic acid (DMEA) in plasma and urine. They reported an increased in all urolithins in urine but only Uro A and C increased in plasma. In addition to Uro A, the author supports the consideration of DMEA as a good biomarker for ellagitannins exposure. Although DMEA may be specific to ellagitannins, it is still difficult to propose DMEA as sole BFI for raspberry intake due to the ubiquitous nature of ellagitannins as they are being shared across many Rubus species, strawberry, walnuts and more. As for urolithins, they have been previously established as strong candidates as part of the multi-metabolite panel for raspberry due to their specificity. These Uros include: Uro A, Uro A glucuronide, Isouro A, Isouro A glucuronide, Uro B, Uro B glucuronide and Uro C. However, Uro A and its derivatives are more likely to be good biomarkers as they are produced by all population, while Isouro A and uro B are not. Despite these Uros being shared with strawberry also as part of its multi-marker panel, the discovery of raspberry aromatic ketones, as well as their sulfate and glucuronide conjugates, help sets apart between the two berries. More efforts are needed to investigate the presence and metabolic fate of raspberry ketones in humans and whether they may be suitable biomarkers for raspberry intake.

Strawberry

Twenty papers were included to investigate the BFIs of strawberry intake in the review of Ulaszewska M. et al. Pelargonidin and urolithin derivatives were suggested as BFIs of strawberry. Besides, the aromatic compounds such as furaneol and mesifurane were also proposed to be used as BFIs for strawberry. However, all these metabolites lack specificity. A multi-BFIs including pelargonidin glucuronides, urolithins and furaneol or mesifurane derivatives was proposed as an adequate biomarker for strawberry intake despite the lack of the authentic standards for pelargonidin glucuronides. In this review, three additional studies investigating biomarkers of strawberry intake were found (33–35). All were chronic interventions (≥ 4 w) that used reconstituted freeze-dried strawberry powder (25-26 g) as treatment. In Zhao et al. (33), a total of 81 bile acids were analyzed in plasma, urine and feces collectively after 4 w of strawberry consumption. Several secondary bile acids, deoxycholic acid, lithocholic acid (and their glycine conjugates) and glycoursodeoxycholic acid have decreased upon intervention. While interesting, it is difficult to recommend primary bile acids as BFIs due to them being internally synthesized (82). Even though secondary bile acids are regarded as bacterial metabolites of the gut microbiota (83), they still lack specificity thanks to their endogenous nature. After 4 weeks of intake, Huang et al. identified 17 plasma phenolic metabolites in hypercholesterolemic patients (34). These include various phenolic acid groups and their derivatives (Table 1) which have already been ruled out as candidates in the previous review for being common metabolites for many fruits and vegetables. In the last study (35), pelargonidin-3-glucuronide (pel-3-gluc) found in both plasma and urine further reinforces its standing as a strong BFI candidate for strawberry as proposed in the previous review alongside Uro A detected in urine samples. In summary, no additional strawberry BFI candidates were found to fit the proposed multi-metabolite panel. Generally, the co-presence of Uros and ACN derivatives in plasma and urine could be considered as an indicator for berry intake. However, sample collection time should be taken into consideration as Uros and ACN have different bioavailability in blood and urine. As mentioned previously, ACN can rapidly degrade and disappear from the circulation within 6 h. Furthermore, the high inter-individual variability of Uros was evident since every metabotype produces uroA but only some metabotype produces isoA or uroB. This ultimately gave rise to its limitation as a singular BFI for berries. (84) As it currently stands, the panel consists of pelargonidin, pelargonidin glucuronide, mesifurane sulfate, furaneol glucuronide and furaneol sulfate. These are reasonably unique to strawberry and they are coupled with the urolithin derivatives to form the proposed multi-BFIs group for strawberry (Table 2).

299 **Blackcurrant**

300 Eight studies investigating the blackcurrant metabolome were discussed in the previous review
301 (17,85–92), with two main ACN identified in urine; cya-3-rut and delphinidin-3-rutinoside. In
302 plasma, these two ACN were also identified beside cya-3-glu and delphinidin-glucoside. There
303 were no bioavailability studies conducted on the other blackcurrant phytochemicals in humans,
304 which shows the need of future clinical trials on blackcurrants and their BFIs. In our review,
305 only one study explored the bioavailability of plasma phenolic acids after an acute single-dose
306 intake of blackcurrant (36). In this study, it was revealed that gallic acid (GA) and
307 protocatechuic acid (PCA) were increased in plasma after blackcurrant intake. However, the
308 participants in this trial were not asked to follow a restricted low polyphenol diet during the
309 trial, and GA and PCA were also detected in their baseline plasma. GA and PCA are phenolics
310 compounds that appears in biofluids samples as a result of gut microbiota metabolism after the
311 consumption of many other fruits and foods (93,94). In line with the previous review, specific
312 BFIs for blackcurrants intake are still lacking, and more future studies to identify new and
313 specific biomarkers of blackcurrant intake are much needed.

314 **Blackberry**

315 Five studies were included in the previous review to examine BFIs of blackberry intake (95–
316 99). Like other Rubus fruits, blackberries are rich in ellagitannins and ACN. Therefore, the
317 metabolites detected in plasma and urine in these studies were derivatives of cyanidins and
318 ellagitannins such as cya-3-gluc, cyanindin-3-glucoside, cya-3-rut, Uro A glucuronide, Uro B
319 glucuronide, ellagic acids. Nevertheless, these metabolites vary between individuals and are
320 not specific to blackberry. The authors suggested to evaluate the Rubus fruits as a group and
321 consider ellagitannins that conjugated with the ester of sanguisorbyl group as BFI. However,
322 clinical trials on the metabolism of these metabolites are scarce and more future human studies
323 are in need. In this review, only one pilot study using LC-MS analysis was conducted on two
324 participants who consumed a blackberry juice as single dose (37). They only detected sulfated
325 cyanidin derivatives in 24 h urine which are not specific to blackberry. Thus, we still need more
326 studies, ideally with untargeted metabolomics, to discover potential biomarker of blackberry
327 intake.
328

329

Table 2: Summary of proposed biomarkers of berry intake

Berries	Suggested BFIs
Blueberries	No specific BFIs. Suggested multi-biomarker panel: Hippuric acid Malvidin glycosides
Cranberries	No specific BFIs. Suggested multi-biomarker panel: Peonidin and cyanidin glycosides ph-γ-VL- sulfate and glucuronide conjugates
Raspberries	Multi-biomarker panel: Raspberry ketone *sulfate*/glucuronide Uro A / Uro A gluc
Strawberries	Multi-biomarker panel: Pel / Pel-3-gluc Uro A / Uro A-gluc Furaneol gluc / Furaneol *sulfate* / Mesifurane *sulfate*
Blackcurrants	No specific BFIs or multi-biomarker panel available.
Blackberries	No specific BFIs or multi-biomarker panel available.

ph-γ-VL: phenyl-γ-valerolactone, Uro A: urolithin A, Uro A gluc: urolithin A glucuronide, Pel: pelargonidin, Pel-3-gluc: pelargonidin-3-glucuronide.

4. Conclusion

This systematic review has managed to provide a comprehensive update upon the previous attempt at unveiling BFIs for berry consumption. The previous review established tentative multi-BFI panels for cranberries, raspberries and strawberries but failed to find suitable metabolite candidates for blackcurrants, blueberries and blackberries. The results through our search are generally aligned with their findings. Additionally, we propose a potential multi-BFI panel for blueberries consisting of hippuric acid and malvidin glycosides, although we appreciated that this multi-biomarker panel is not specific enough for blueberries, and intact anthocyanins are not great candidates as biomarkers due to the low concentrations in plasma and urine and the fact that they are highly unstable. As a whole, the BFI panels for cranberries, raspberries and strawberries remain intact while BFIs specific to blackcurrants and blackberries

345 are yet to be found. In conclusion, no specific biomarkers of intake for berries or individual
346 berries currently exist. The multi-biomarker approach is currently still the best option for
347 estimating intake. However, more robust RCTs, with metabolomics in particular, are much
348 encouraged for biomarker discovery. The lack of authentic standards for accurate metabolite
349 quantification continues to be a hindrance and this issue should be prioritised as well.

350

351 **Authors contributions**

352 HM and ARM designed the systematic search. HM and AC drafted the first version of the
353 manuscript. All the authors critically revised the draft and approved the final version.

354 **Notes**

355 The authors declare no conflict of interest.

356 **Funding**

357 The study was supported by Grant PID2020-114921RB-C21 and PID2021-128542OA-I00
358 funded by MCIN/AEI/ 10.13039/501100011033 and CIBERFES, CB16/10/00269, from the
359 Instituto de Salud Carlos III all of them by "ERDF A way of making Europe". The Generalitat
360 de Catalunya's Agency AGAUR of 2021SGR00687. *Maria de Maeztu Unit of Excellence grant*
361 *(CEX2021-001234-M)* funded by *(MICIN/AEI/FEDER, UE)*. CAL thanks ICREA Academia
362 Award. HM thanks the scholarship and travel grants from the University of Barcelona (APIF)
363 and Institut de Nutrició i Seguretat Alimentària (INSA) de la Universitat de Barcelona, for the
364 scholarship and travel grants.

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Results

Supplementary Table 1: Keywords used for the search in Ulaszewska M. et al. systematic review

Database / keywords	(Fruit name* OR botanical name), AND (urine or plasma or serum or excretion or blood) AND (human* OR men OR women OR patient* OR volunteer* OR participant*) AND (biomarker* OR marker* OR metabolite* OR biokinetics OR biotransformation OR pharmacokinetics OR bioavailability OR ADME) AND (intake OR meal OR diet OR ingestion OR administration OR consumption OR eating OR drink*)
PubMed	All fields
Web of Science	Topic
Scopus	Article Title/Abstract/Keywords

5.2. Nutritional Intervention Clinical Trial: ATTACH study

5.2.1. Publication 2. Influence of Plasma-Isolated Anthocyanins and Their Metabolites on Cancer Cell Migration (HT-29 and Caco-2) In Vitro: Results of the ATTACH Study.

Inken Behrendt, Isabella Röder, Frank Will, **Hamza Mostafa**, Raúl Gonzalez-Dominguez, Tomás Meroño, Cristina Andres-Lacueva, Mathias Fasshauer, Silvia Rudloff and Sabine Kuntz. *Antioxidants journal*. 2022, 11(7), 1341; https://doi.org/10.3390/antiox11071341

Journal impact factor: 7.675

Journal quartile/decile: Q1/D1 (Category: Food Science & Technology, 12/144)

Abstract:

Cancer mortality is mainly due to metastasis. Therefore, searching for new therapeutic agents suppressing cancer cell migration is crucial. Data from human studies regarding effects of anthocyanins on cancer progression, however, are scarce and it is unclear whether physiological concentrations of anthocyanins and their metabolites reduce cancer cell migration in vivo. In addition, interactions with chemotherapeutics like 5-fluorouracil (5-FU) are largely unknown. Thus, we combined a placebo-controlled, double-blinded, cross-over study with in vitro migration studies of colon cancer cell lines to examine the anti-migratory effects of plasma-isolated anthocyanins and their metabolites (PAM). Healthy volunteers (n = 35) daily consumed 0.33 L of an anthocyanin-rich grape/bilberry juice and an anthocyanin-depleted placebo juice for 28 days. PAM were isolated before and after intervention by SPE. HT-29 and Caco-2 cells were incubated with PAM in a Boyden chamber. Migration of HT-29 cells was significantly inhibited by PAM from juice but not from placebo. In contrast, Caco-2 migration was not affected. Co-incubation with 5-FU and pooled PAM from volunteers (n = 10), which most effectively inhibited HT-29 migration, further reduced HT-29 migration in comparison to 5-FU alone. Therefore, PAM at physiological concentrations impair colon cancer cell migration and may support the effectiveness of chemotherapeutics.

Resumen:

La mortalidad por cáncer se debe principalmente a la metástasis. Por lo tanto, la búsqueda de nuevos agentes terapéuticos que supriman la migración de células cancerosas es crucial. Sin embargo, los datos de estudios humanos sobre los efectos de las antocianinas en la progresión del cáncer son escasos y no está claro si las concentraciones fisiológicas de antocianinas y sus metabolitos reducen la migración de células tumorales in vivo. Además, las interacciones con quimioterapéuticos como el 5-fluorouracilo (5-FU) son en gran parte desconocidas. Por lo tanto, combinamos un estudio cruzado, doble ciego y controlado con placebo con estudios de migración in vitro utilizando líneas celulares de cáncer de colon para examinar los efectos anti-migratorios de las antocianinas aisladas del plasma y sus metabolitos (PAM). Los voluntarios sanos (n = 35) consumieron diariamente 0,33 L de un zumo de uva/arándano rico en antocianinas y un zumo de placebo sin antocianinas durante 28 días. Las PAM se aislaron antes y después de la intervención mediante extracción en fase sólida (SPE). Las células HT-29 y Caco-2 se incubaron con PAM en una cámara de Boyden. La migración de las células HT-29 fue inhibida significativamente por las PAM del zumo, pero no por las del placebo. En cambio, la migración de Caco-2 no se vio afectada. La co-incubación con 5-FU y PAM combinadas de los voluntarios (n = 10), que inhibieron de manera más efectiva la migración de HT-29, redujo aún más la migración de HT-29 en comparación con el 5-FU solo. Por lo tanto, las PAM a concentraciones fisiológicas afectaronn la migración de las células de cáncer de colon y podrían apoyar la efectividad de los quimioterapéuticos.

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Influence of Plasma-Isolated Anthocyanins and Their Metabolites on Cancer Cell Migration (HT-29 and Caco-2) In Vitro: Results of the ATTACH Study

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Citation: Behrendt, I.; Röder, I.; Will, F.; Mostafa, H.; Gonzalez-Dominguez, R.; Meroño, T.; Andres-Lacueva, C.; Fasshauer, M.; Rudloff, S.; Kuntz, S. Influence of Plasma-Isolated Anthocyanins and Their Metabolites on Cancer Cell Migration (HT-29 and Caco-2) In Vitro: Results of the ATTACH Study. *Antioxidants* **2022**, *11*, 1341. https://doi.org/10.3390/antiox11071341

Academic Editor: Hae-Jeung Lee

Received: 31 May 2022
Accepted: 5 July 2022
Published: 8 July 2022

Abstract: Cancer mortality is mainly due to metastasis. Therefore, searching for new therapeutic agents suppressing cancer cell migration is crucial. Data from human studies regarding effects of anthocyanins on cancer progression, however, are scarce and it is unclear whether physiological concentrations of anthocyanins and their metabolites reduce cancer cell migration in vivo. In addition, interactions with chemotherapeutics like 5-fluorouracil (5-FU) are largely unknown. Thus, we combined a placebo-controlled, double-blinded, cross-over study with in vitro migration studies of colon cancer cell lines to examine the anti-migratory effects of plasma-isolated anthocyanins and their metabolites (PAM). Healthy volunteers (n = 35) daily consumed 0.33 L of an anthocyanin-rich grape/bilberry juice and an anthocyanin-depleted placebo juice for 28 days. PAM were isolated before and after intervention by solid-phase extraction. HT-29 and Caco-2 cells were incubated with PAM in a Boyden chamber. Migration of HT-29 cells was significantly inhibited by PAM from juice but not from placebo. In contrast, Caco-2 migration was not affected. Co-incubation with 5-FU and pooled PAM from volunteers (n = 10), which most effectively inhibited HT-29 migration, further reduced HT-29 migration in comparison to 5-FU alone. Therefore, PAM at physiological concentrations impairs colon cancer cell migration and may support the effectiveness of chemotherapeutics.

Keywords: anthocyanins; migration; intervention study; colon cancer; 5-fluorouracil; grapes; bilberry; antioxidant capacity; juice

1. Introduction

Colorectal cancer (CRC) is one of the most common cancer types worldwide. In 2020, more than one million patients were newly diagnosed and ~600,000 CRC deaths occurred [1]. However, CRC incidence shows distinct geographic variations, with higher incidence rates in industrialized countries revealing Western lifestyle, and particularly Western diet, as major modifiable risk factors [2,3]. Although the 5-year relative survival rate for CRC patients with localized disease is about 90% [4], metastasis is associated with poor outcomes. In CRC patients with metastasis, the 5-year relative survival rate

dramatically declines to 14% [4]. Therefore, CRC is the second leading cause of cancer death globally [1] and searching for new therapeutic agents to impair tumor progression and metastasis is critical.

Carcinogenesis and metastasis are associated with oxidative stress that is characterized by excessive levels of reactive oxygen species (ROS) [5]. ROS are highly reactive and capable of damaging macromolecules such as DNA, proteins, and lipids, as well as cellular structures, thus promoting malignant transformation [5,6]. In addition, mitochondrial dysfunction and high ROS levels increment the migratory and invasive potential of several cancer cell lines [7–9]. Therefore, antioxidants are hypothesized as chemopreventive and chemotherapeutic agents [6]. In recent years, several plant-derived phytochemicals, particularly polyphenols, have attracted remarkable attention for their potential to prevent tumor initiation, promotion, and progression, due to their low cost, low toxicity, and the undesirable adverse side effects of chemotherapeutical drugs [10]. Anthocyanins, a subgroup of flavonoids, belong to the most prevalent group of polyphenols in fruits [11] and are responsible for their orange to bluish-red color [12–14]. Pelargonidin, cyanidin, delphinidin, peonidin, petunidin, and malvidin are the predominant dietary anthocyanidins. These six anthocyanidins account for more than 90% of all yet identified anthocyanins [15]. However, anthocyanins in fruits are primarily present as glycosides or acylated glycosides. Berries such as blueberries, blackberries, raspberries, cranberries, and grapes show anthocyanin contents between 21 and 390 mg per 100 g fresh weight [16] with peonidin and cyanidin being the major anthocyanins in grapes and berries, respectively [17]. It is well known, that due to their structural polyphenolic characteristics, anthocyanins exhibit a high antioxidative potential, e.g., because of their capability for donating electrons [18], scavenging ROS [19,20], preventing ROS-induced oxidative damage or influencing antioxidative enzyme expression [21,22]. In epidemiological studies, total dietary anthocyanin intake is inversely associated with CRC [18,23,24]. In addition, numerous in vitro and in vivo studies reveal that anthocyanins are able to decelerate CRC promotion and progression by several mechanisms such as triggering cell cycle arrest and apoptosis or inhibiting proliferation and invasion by different signaling pathways [10,17], whereas studies regarding the anti-migratory effects of anthocyanins on colon cancer cells are scarce [25–27]. Nonetheless, it is also known that the bioavailability of anthocyanins is relatively low and native anthocyanins are only detectable at very low concentrations in the systemic circulation [28,29]. Anthocyanins which are not absorbed in the duodenum reach the lower gastrointestinal tract where they are metabolized by the gut microbiota. Recently, it has been shown that microbially metabolized anthocyanin compounds account for the majority of absorbed berry phenols [30]. Moreover, these anthocyanin metabolites also show bioactive properties and seem to be more effective than native anthocyanins to reduce Caco-2 cell proliferation [17]. In this context, we have recently shown for the first time that physiological concentrations of anthocyanins and their metabolites isolated from the plasma of healthy volunteers after a single dose of an anthocyanin-rich juice were able to reduce tumor cell migration of the pancreatic cancer cell line PANC-1 in vitro [31]. This was accompanied by a significant reduction in ROS levels and decreased matrix metalloproteinases (MMP-2 and MMP-9), as well as NF-κB, mRNA expression [31]. Despite the common concept of antioxidants as tumor suppressors, recent evidence indicates that antioxidants may also act as tumor promotors, especially in metastasis [6]. Therefore, the role of ROS and antioxidants such as anthocyanins during metastasis are still not fully understood [32]. Similarly, the prognostic importance of enzymatic and non-enzymatic biomarkers of oxidative stress in colorectal cancer progression and metastasis is still unclear. In CRC patients biomarkers of oxidative stress are significantly increased, whereas the total antioxidant capacity (TAC) is significantly lower compared with healthy controls [5,33]. Thus, CRC patients may be more vulnerable to oxidative stress and administering of anthocyanins might improve the antioxidant capacity, overall health, and the outcome for CRC patients.

Therefore, the primary outcome of the present study was to determine whether physiological concentrations of anthocyanins and their metabolites, isolated from plasma of healthy volunteers after long-term consumption of an anthocyanin-rich grape/bilberry juice, impair the migratory potential of two colon cancer cell lines, HT-29 and Caco-2, in vitro. Although both cell lines were isolated from adenocarcinoma and exhibit epithelial phenotypes [34–36], HT-29 and Caco-2 cells were chosen due to their varying grade of differentiation [37] and their different migratory, as well as metastatic, potential [36]. We further aimed to investigate whether possible anti-migratory effects were associated with alterations of antioxidant status parameters in the plasma or urine of the volunteers (secondary outcome).

2. Materials and Methods

2.1. Preparation and Characterization of the Anthocyanin-Rich Juice and the Anthocyanin-Depleted Placebo

The anthocyanin-rich juice and the anthocyanin-depleted placebo were produced at the Hochschule Geisenheim University (Department of Beverage Research, Geisenheim, Germany) and were similar to the juices for the ANTHONIA study with minor modifications [21,38]. Briefly, juices were made from 80% red grape juice (grape variety *Accent*) and 20% bilberry juice (Heidelbeersaft blank BIO (Bayernwald KG, Hengersberg, Germany). Grapes were extracted in a press and the resulting juice was separated, blended with the bilberry juice, pasteurized, and hot-filled into 0.33 L brown glass bottles. Placebo juice was obtained by passing the juice through SP70 Sepabeads® absorber resin (Resindion S.r.l., Binasco, Italy). Both juices were analyzed directly after membrane filtration (0.45 µm) for basic analytical parameters such as total phenolics, concentrations of anthocyanins, and TEAC (Trolox equivalent antioxidative capacity) as described elsewhere [39]. Anthocyanins were analyzed by LC–MS as previously described [21]. Quantitation was carried out in duplicate using peak areas detected at 520 nm and based on external calibration via the reference substance cyanidin-3-O-glucoside (0.1–100 mg/L; linearity of calibration, $r^2 = 0.9999$). For cyanidin-3-O-glucoside, the limit of detection was 0.01 mg/L and the limit of quantitation was 0.04 mg/L.

2.2. Study Design and Study Subjects

The randomized, placebo-controlled, double blinded, cross-over ATTACH study (Anthocyanins Target Tumor cell Adhesion—Cancer vs. Endothelial Cell (HUVEC) Interactions study) was carried out at the Department of Nutritional Science, Justus Liebig University, Giessen (Germany) between April and August 2019. Sample size was calculated based on the results of our previous published migration study [31] with a β- and α-error of 0.8 and 0.05 and a drop-out rate of 20%. Calculations were made with Stata Version 15.1 (StataCorp LLC, College Station, TX, USA) from ASKNET solutions AG (Karlsruhe, Germany). In total, 45 healthy students from the Justus Liebig University, Giessen were recruited and due to exclusion criteria (drugs and antibiotics in the last 3 months before the study, vitamin and mineral supplementation, as well as intestinal or cardiovascular diseases) 2 were excluded. After randomization 8 declined to participate due to non-compliance with the nutritional recommendations. From the 35 volunteers (female $n = 27$ and male $n = 8$), 13 were omnivores, 8 vegetarians, 2 vegans and 1 pescetarian with a mean ± SD age of 24.4 ± 2.3 years (range: 19–29 years), an initial body weight of 64.3 ± 17.9 kg (range: 49–93 kg), and a BMI of 21.7 ± 2.6 kg/m^2 (range: 18.4–27.6 kg/m^2). Baseline characteristics did not change during the total study period. Of the 35 subjects, 34 collected all samples (blood and urine), whereas one subject had an incomplete urine sample collection.

Volunteers received the beverages, denoted as "one" or "two", weekly at the Department of Nutritional Science. The beverages were filled in brown bottles to ensure blinding and distributed weekly to the volunteers by the lab personal. Participants were instructed to daily consume 0.33 L of the anthocyanin-rich juice or the anthocyanin-depleted placebo for 28 days. They were instructed to keep the juices cool and to avoid their exposure to

direct light. Thus, after a 7-day wash-out period, a 28-day intervention period followed, and the first phase was completed by a 14-day run-out period. After the run-out phase, the next phase started with the next beverage (Figure 1).

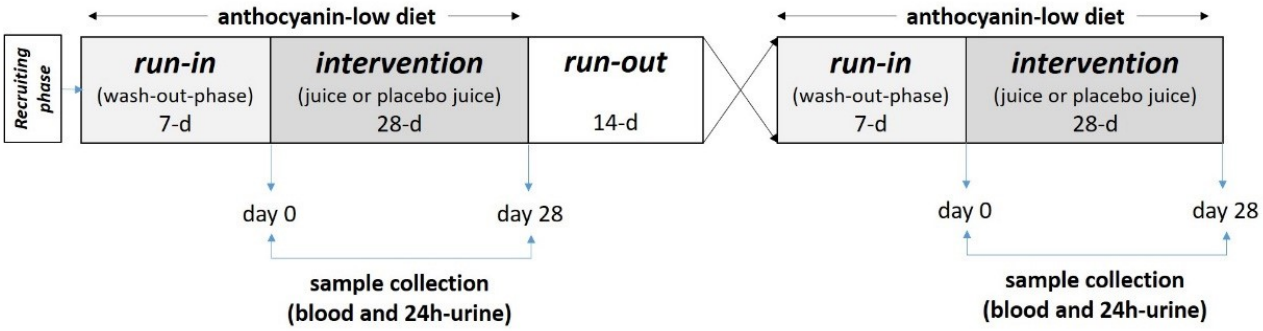


Figure 1. Study design of the ATTACH study. Participants consume the anthocyanin-rich juice and the anthocyanin-depleted placebo. Before (day 0) and after each intervention (day 28), blood and 24 h-urine samples were collected and processed for biochemical analyses (n = 35). d, day.

As phenolic compounds including anthocyanins are present in many foodstuffs and beverages, participants were explicitly counseled prior to the start of the study to follow a low phenolic/anthocyanin diet during the run-in and intervention period to avoid possible effects of other phenolics, especially anthocyanins, from the diet. Similar to our ANTHONIA study, the volunteers received a list of categorizing foodstuffs including beverages into "quantitatively limited" and "not being allowed" with slight modifications [21]. Foodstuffs were categorized according to their anthocyanin content based on data using the USDA Database for the Flavonoid Content of Selected Foods (release 3.3 (2018); http://www.ars.usda.gov/, accessed on 1 March 2019) or a database on polyphenol contents in food (Polyphenol-Explorer, release 3.0; http://www.phenol-explorer.eu/compounds, accessed on 1 March 2019) (Table S1).

Participants were also instructed to record their dietary intake over 3 days during each intervention period in order to estimate their daily energy and nutrient intakes. Dietary records were analyzed using the DGE-PC Professional software, version 1.10.0.0 (Table S2). Participants were instructed to maintain their usual physical activity.

The study protocol was approved by the local ethics committee (registration number 13/10) and according to the guidelines laid down in the Declaration of Helsinki. It is registered at DRKS (Deutsche Register Klinischer Studien) with the registration number DRKS00014767. Written informed consent was obtained from all included participants and data collection was conducted by the Department of Nutritional Science (Giessen, Germany).

2.3. Collection of Plasma and Urine Samples

Sample collection occurred before and after the two intervention periods. After an initial 7-day run-in period, participants were instructed to collect their 24 h-urine and blood was drawn by venipuncture into tubes with EDTA as anticoagulant (Sarstedt & Co., Nuembrecht, Germany). Plasma was separated immediately by centrifugation (1200× g for 15 min; 4 °C). The supernatant was divided into aliquots and stored at −80 °C until assayed (day 0). Volunteers again collected their 24 h-urine at the end of the intervention and consumed the juice after an overnight fast together with breakfast on the last day of the intervention period. Plasma samples were collected 6 h after juice ingestion (day 28).

2.4. Isolation of Plasma Anthocyanins and Their Metebaolites by Solid Phase Extraction

Plasma extraction of anthocyanins and their metabolites was based on the method described recently [40]. Briefly, each aliquot (1 mL of plasma acidified with 30 µL of 50% aqueous formic acid (Lgc Promochem, Wesel, Germany)) was loaded onto an Oasis-HLB (1 mL/30 mg) SPE cartridge (Waters, Inc., Eschborn, Germany), preconditioned with

1 mL of methanol (Thermo Fischer Scientific, Langenselbold, Germany) and 1% formic acid, followed by 1 mL of acidified water (1% formic acid). The cartridge was then washed with 1 mL of acidified water, after which anthocyanins and their metabolites were eluted with 1 mL of acidified methanol. Afterwards, eluates were dried under N_2 for approximately 3 h [40]. For cell migration studies with HT-29 and Caco-2 cells, dried anthocyanins isolated from plasma as well as their metabolites (PAM) were resolved in the same volume of culture media (1 mL; pH 7.2) as the original plasma volume (1 mL).

2.5. Cell Culture

The two human colon carcinoma cell lines HT-29 (HTB-38) and Caco-2 (HTB-37) were purchased from the American Type Culture Collection (ATCC) (San Diego, CA, USA). HT-29 is a cell line with epithelial morphology that was isolated in 1964 from a primary tumor obtained from a 44-year-old female patient with colorectal cancer by J. Fogh [34]. HT-29 cells were grown in RPMI 1640 GlutaMax (Invitrogen GmbH, Darmstadt, Germany) supplemented with 1 mmol/L sodium pyruvate (Invitrogen GmbH, Darmstadt, Germany) and 10% fetal calf serum (FCS) (Invitrogen GmbH, Darmstadt, Germany). Caco-2 are epithelial cells isolated from colon tissue derived from a 72-year-old male with colorectal adenocarcinoma and has been widely used as a model of the intestinal epithelial barrier [35]. Cells were grown in DMEM (Invitrogen GmbH, Darmstadt, Germany) supplemented with 5 mM L-glutamine (Invitrogen GmbH, Darmstadt, Germany), 1 mmol/L sodium pyruvate and 10% FCS. Thus, both cell lines were isolated from colon adenocarcinomas and were most widely used for in vitro studies to compare their tumorigenicity genotype [41–43]. Both cell lines were sub-cultured twice a week, incubated at 37 °C in a humidified 5% (*v*/*v*) CO_2 atmosphere and used between passages 10 to 35. Culture medium was changed every two days.

2.6. Cell Migration of Colon Cancer Cells In Vitro

Tumor cell migration was assessed in a Boyden chamber with the use of the CytoSelect 24-well Cell Migration Assay (CellBiolabs, San Diego, CA, USA) according to earlier studies [31]. The feeder trays were coated with 100 µL of 10 µg/mL collagen (Merck GmbH, Darmstadt, Germany) and aspirated until dryness. Onto the upper side of the 24-well feeder chamber (diameter of the chamber 6.5 mm; pore size 8 µm), cells at a density of 1×10^5/mL were seeded in DMEM or RPMI 1640 GlutaMax containing 1% FCS and diluted PAM (day 0 vs. day 28), whereas DMEM or RPMI 1640 GlutaMax supplemented with 10% FCS were added to the lower chamber [41]. Cells were incubated in the feeder tray for 36 h at 37 °C. Then cells on the lower side were detached from the membrane using a cell detachment solution and afterwards lysed with a fluorescent-dye-containing buffer. The extent of migration was assessed by the intensity of the fluorescence signal, which was measured with a Synergy H1 microplate fluorescence reader (Biotek, Karlsruhe, Germany). The number of migrated cells was determined according to a calibration curve (500 to 15,000 cells). Results are expressed as median with interquartile range from $n = 35$ or $n = 34$ volunteers. A 100 mM stock of 5-FU (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) was prepared in absolute DMSO (Merck GmbH, Darmstadt, Germany) and stored at −20 °C. The concentration of DMSO was less than 1% of drug treatment. For treatment, 5-FU was diluted in culture media and added to cultures to give the desired final concentration.

2.7. Assessment of Cell Viability by Flow Cytometry

Viability of cells was determined by flow cytometry with the Guava® ViaCount™ reagent (Luminex, MV's, Hertogenbach, Netherlands). Therefore, HT-29 cells were seeded at a density of 1×10^5 cells/mL in 24-well plates in complete medium with or without PAM from the anthocyanin-rich juice, which were isolated before or after the 28-day intervention as well as with different concentrations of 5-FU. After 36 h incubation, cells were washed twice with PBS (Invitrogen GmbH, Darmstadt, Germany) and trypsinized with

TrypLE Express (Invitrogen GmbH, Darmstadt, Germany). Cell viability and cytotoxicity were measured according to the manufacturer's instructions using a Guava® Muse® Cell Analzyer (Luminex, MV'ss, Hertogenbach, Netherlands).

2.8. Oxidative Biomarkers in Plasma and Urine Samples

Oxidative biomarkers in plasma and urine samples were measured before (day 0) and after each intervention period (day 28). Enzyme activities of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) were determined by colorimetric methods according to the manufacturer's instructions (Cayman Chemical Company, Ann Arbor, MI, USA) in a Synergy H1 microplate fluorescence reader (Biotek, Karlsruhe, Germany) as described previously [21]. The amount of water and lipid soluble antioxidants in plasma was assessed using the ABTS (2,2-azino-di-3-ethylbenzthiazoline sulphonate) assay (Cayman Chemical Company, Ann Arbor, MI, USA). Generation of $ABTS^+$ from ABTS by metmyoglobin is inhibited by antioxidants and yields a reduction in absorbance at 405 nm. The capacity of antioxidants to reduce $ABTS^+$ generation was compared with that of Trolox and quantified as Trolox equivalents (mmol/L TEAC) [21]. All analyses were performed in duplicate and intra-assay coefficients of the assays were <12%. The total phenolic content (TPC) in urine samples was determined after solid-phase extraction (SPE) (Oasis® MAX 96-well plate cartridges) using a rapid Folin–Ciocalteu method described earlier [44,45]. Briefly, in a thermo microtiter 96-well plate, 170 µL of Milli-Q water, 15 µL of the urine extract, 12 µL of the Folin–Ciocalteu reagent (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) and 30 µL of 20% sodium carbonate were mixed. After 1 h of incubation at room temperature in the dark, the absorbance was measured at 750 nm using an UV/VIS Thermo Multiskan Spectrum spectrophotometer (Vantaa, Finland). Gallic acid (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) was used as a standard and quantified as gallic acid equivalents (mg/L). All samples were processed in triplicate and the coefficient of variation (CV) was <10%. Results were multiplied by the urine output and expressed as gallic acid equivalents (GAE) in mg/24 h

2.9. Statistical Analyses

Data from the volunteers who completed all phases of the study (n = 35; n = 34) were analyzed. The outcome measures were prospectively designated as the differences in migration of HT-29 and Caco-2 cells in vitro (primary outcome) and antioxidative parameters (secondary outcome) of placebo and juice treatment before (day 0) and after (day 28) intervention. Before-treatment versus after-treatment data within groups were analyzed using a repeated measures one-way ANOVA, with Šídák's post hoc test. A mixed model with multiple comparison test (Šídák's) were used for data sets with missing values. The normality of continuous variables was assessed using Kolmogorov–Smirnov normality test. Asterisks are used in the figures to denote p values < 0.05, which were considered significant. Data were expressed as mean ± SD or as median with interquartile range (25th–75th percentile). Correlation analyses were evaluated using Spearman correlation (r) with differences between values from day 28 and day 0 after anthocyanin-rich juice intake (e.g., Δ values = values J_28-days—values J_0-days). GraphPad Prism 9 Version 9.3.1. from ASKNET solutions AG (Karlsruhe, Germany) was used for data analyses.

3. Results

3.1. Composition of the Anthocyanin-Rich Juice and the Anthocyanin-Depleted Placebo

In this cross-over, placebo-controlled intervention study, an anthocyanin-rich grape/bilberry juice was given to healthy male and female volunteers for 28 days in order to investigate the influence of PAM on cancer cell migration in vitro. To eliminate possible effects of other juice compounds, an anthocyanin-depleted placebo juice was also applied. The anthocyanin-rich juice exhibited an especially high antioxidant activity corresponding to a higher TEAC value (27 ± 1.7 mmol/L), as well as higher concentrations of total phenolics (2622 ± 56 mg/L catechin equivalents) and anthocyanins (942 ± 10 mg/L

cyanidin-3-O-equivalents) in comparison with the anthocyanin-depleted placebo (TEAC: 1.0 ± 0.04 mmol/L; total phenolics: 115 ± 5.0 mg/L catechin equivalents; anthocyanins: 6.3 ± 0.5 mg/L cyanidin-3-O-glucoside equivalents). In comparison with commercially available grape (TEAC: 10–12 mmol/L) and blueberry juices (TEAC: 13–17 mmol/L) the TEAC value of our juice was approximately 2–3-fold higher [46–48]. Regarding anthocyanins, which were the main phenolics in both beverages, the applied beverages considerably differed in their profile and contents (Table 1). Due to the high grape content, peonidin-3,5-O-diglucoside and malvidin-3,5-O-diglucoside were the most abundant anthocyanins in the juice and accounted for more than 51.5% of all anthocyanins, followed by peonidin-3-O-glucoside and malvidin-3-O-glucoside. In comparison with the juice, the placebo contained only minor amounts of anthocyanins.

Table 1. Anthocyanin composition of the anthocyanin-rich juice and the anthocyanin-depleted placebo [1].

	Anthocyanin-Rich Juice		Anthocyanin-Depleted Placebo	
Anthocyanins	**(mg/L)**	**(%)**	**mg/L**	**(%)**
peonidin-3,5-O-diglucoside	346 ± 12.5	36.8	1.7 ± 0.02	26.9
malvidin-3,5-O-diglucoside	138 ± 8.4	14.7	0.88 ± 0.06	14.0
peonidin-3-O-glucoside	83.5 ± 6.4	8.9	0.37 ± 0.01	5.9
malvidin-3-O-glucoside	63.4 ± 3.8	6.7	0.30 ± 0.01	4.7
delphinidin-3-O-glucoside	61.5 ± 2.9	6.5	0.80 ± 0.03	12.7
delphinidin-3-O-galactoside	53.6 ± 0.6	5.7	0.75 ± 0.01	11.9
delphinidin-3-O-arabinoside	53.4 ± 1.6	5.7	0.56 ± 0.02	7.4
petunidin-3-O-glucoside	43.7 ± 1.9	4.6	0.37 ± 0.02	5.8
cyanidin-3-O-arabinoside	27.2 ± 1.7	2.9	0.11 ± 0.02	1.7
cyanidin-3,5-O-diglucoside	18.2 ± 1.6	1.9	0.29 ± 0.00	4.6
malvidin-3-(6″-O-coumaryl)-5-O-diglucoside	17.1 ± 0.4	1.8	n.d.	n.d.
petunidin-3-O-galactoside	13.2 ± 0.3	1.4	0.11 ± 0.01	1.8
petunidin-3-O-arabinoside	8.8 ± 0.0	0.9	0.05 ± 0.01	0.8
malvidin-3-O-arabinoside	5.3 ± 0.0	0.6	0.02 ± 0.00	0.3
peonidin-3-O-galactoside	4.3 ± 0.0	0.5	0.01 ± 0.00	0.1
delphinidin-3,5-O-diglucoside	3.4 ± 0.0	0.4	0.09 ± 0.01	1.4
Sum	942 ± 10	100	6.3 ± 0.5	100

[1] Juices were analyzed by LC–MS ($n \geq 2$) and data are expressed as mean ± SD mg cyanidin-3-O-glucoside equivalents per L.n.d., non-detectable

3.2. Influence of Plasma Anthocyanins and Their Metabolites on HT-29 and Caco-2 Colon Cancer Cell Migration

Anthocyanins and their metabolites were extracted by SPE from plasma samples before (0 d) and after the intervention (28 d) with juice or placebo. Afterwards, dried PAM were solved in cell culture media and applied to the two colon cancer cell lines HT-29 and Caco-2. As shown in Figure 2, cancer cell migration was differently affected by incubation with PAM from both beverages. In case of HT-29 cells, PAM from the juice significantly reduced cancer cell migration from 3806 (3646–4013) to 3453 (3052–3787) cells per cavity ($p < 0.001$). In comparison, no inhibition was observed after exposure to PAM from the placebo (Figure 2a). In contrast to HT-29 cells, migration of Caco-2 cells was neither influenced by PAM from the placebo nor by PAM from the juice (Figure 2b). Therefore, this study shows that physiological concentrations of PAM significantly impair the migratory potential of HT-29 cells, whereas migration of Caco-2 cells was not affected.

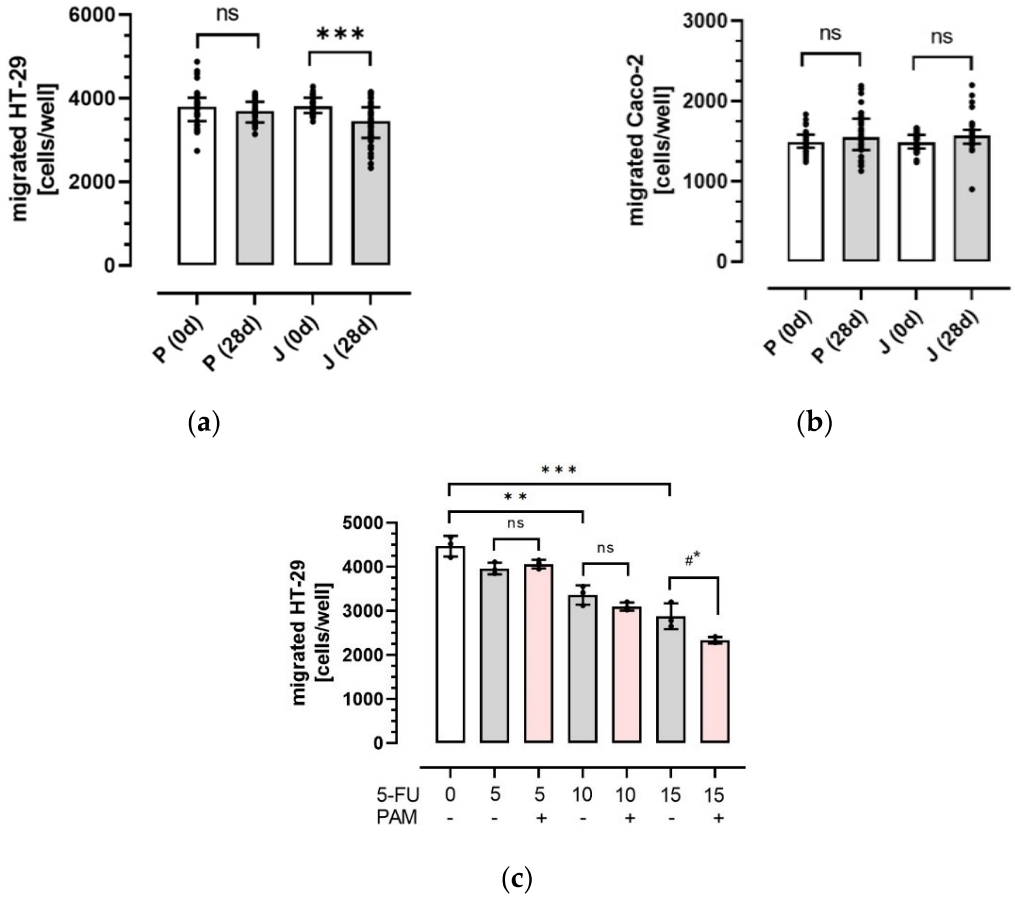
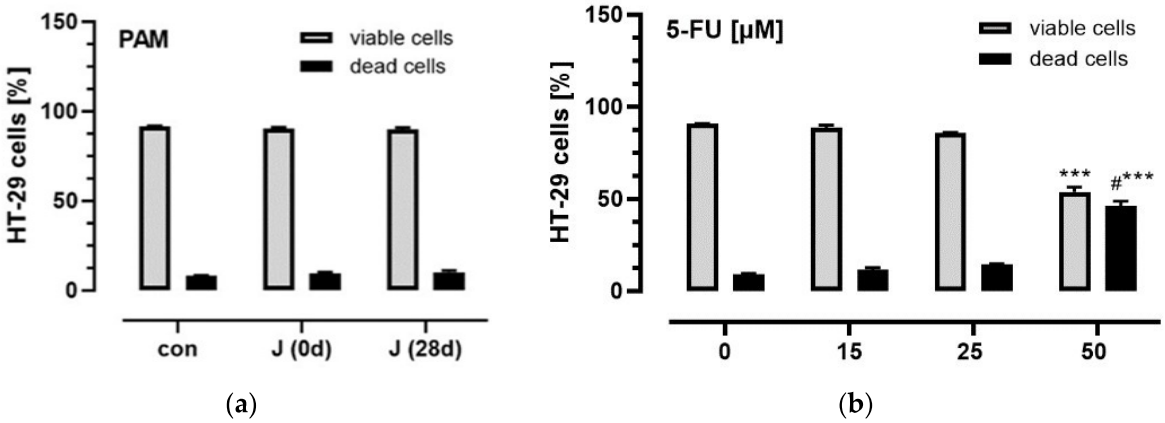


Figure 2. Migration of HT-29 and Caco-2 cells in vitro. HT-29 (**a**) and Caco-2 (**b**) cells were incubated with PAM from the anthocyanin-depleted placebo (P; $n = 34$) and anthocyanin-rich juice (J; $n = 35$) that were isolated before (0 d) and after 28-day (28 d) intervention. Migration was studied in a Boyden chamber with collagen-coated transwells. Basal cells in the lower chamber were measured after 36 h and migrated cell counts were detected fluorometrically as described in the Methods section. HT-29 cells (**c**) were exposed to indicated concentrations of 5-FU alone (5, 10, and 15 µM) and with pooled PAM ($n = 10$; most effective from (**a**)). Values are presented as aligned dot plots with median and interquartile range (25th–75th) (**a**,**b**) or means with standard deviation (**c**). Significant differences were calculated with a mixed model with multiple comparison test (Šídák's) or ANOVA with multiple comparison test (Šídák's). Values were different with ** $p < 0.01$ and *** $p < 0.001$ compared with the corresponding controls or with #* $p < 0.05$ compared with 5-FU (15 µM) alone. ns, non significant.

To examine the effect of 5-fluorouracil (5-FU) on HT-29 cell migration, various concentrations of 5-FU were applied. 5-FU is a common chemotherapeutic agent in colorectal cancer [49,50] and it is known that flavonoids can interact with chemotherapeutic agents [51]. However, possible interactions between anthocyanins and 5-FU are largely unknown. As shown in Figure 2c, HT-29 cell migration was concentration-dependently inhibited by 5-FU treatment. Compared with untreated control cells, incubation of HT-29 cells with the most effective 5-FU concentration (15 µM) significantly reduced colon cancer cell migration from 4472 ± 231 to 2882 ± 289 cells per cavity ($p < 0.001$). To further investigate, possible synergistic or even antagonistic effects of PAM on 5-FU treatment, HT-29 cells were co-incubated with 5-FU and pooled PAM from volunteers ($n = 10$) that most effectively inhibited HT-29 cancer cell migration. In comparison with 5-FU alone (15 µM), co-incubation with pooled PAM further decreased HT-29 cell migration from 2882 ± 289 to 2338 ± 71 cells per cavity ($p < 0.05$), respectively (Figure 2c). These results indicate that physiological concentrations of anthocyanins and their metabolites may promote the effects of classical chemotherapeutic agents like 5-FU.

3.3. Influence of Plasma Anthocyanins and Their Metabolites on HT-29 and Caco-2 Cell Viability

To investigate possible cytotoxic effects of PAM on colon cancer cells, HT-29 were incubated with the pooled PAM from volunteers that most effectively inhibited HT-29 cancer cell migration (n = 10). As shown in Figure 3 viability of HT-29 cells was neither affected by incubation with PAM for 36 h, nor by 5-FU in low doses (≤25 µM). In contrast, 50 µM 5-FU significantly decreased viability of HT-29 cells compared with controls. In addition, PAM showed no cytotoxic effects in non-tumor cells (Figure S1).



(**a**) (**b**)

Figure 3. Effects of PAM (**a**) and 5-FU (**b**) on HT-29 cell viability. HT-29 cells were seeded at a density of 1 × 10^5 cells/mL in 24-well plates in complete medium with or without PAM from the anthocyanin-rich juice that were isolated before (J (0 d)) or after 28-day intervention (J (28 d)) or with medium alone (con), as well as with different concentrations of 5-FU. After 36 h incubation, cells were washed twice with PBS, trypsinized and cell viability was measured using a Guava® Muse® Cell Analyzer. Data are expressed as bars [%] with standard deviation. Significant differences were calculated with ANOVA with multiple comparison test. Values were different with *** $p < 0.001$ compared with viable cells of the controls (con) or with #*** $p < 0.001$ compared with dead cells of the controls (n = 2).

3.4. Effects of the Anthocyanin-Rich Juice and the Anthocyanin-Depleted Placebo on Antioxidative Biomarkers in Plasma and Urine

Biomarkers of oxidative stress are significantly increased in CRC patients, whereas TAC is significantly lower compared with healthy controls [5,33]. Thus, we aimed to investigate whether long-term consumption of an anthocyanin-rich juice improves the antioxidant status of healthy volunteers. A significant increase of median (25th–75th percentile) plasma SOD activity from 7.53 (6.27–9.55) to 9.52 (7.21–10.69) U/mL ($p < 0.001$) was observed after a 28-day intervention with the juice. In contrast, no change in plasma SOD activity was found after consumption of the placebo (Figure 4a). Similarly, after ingestion of the anthocyanin-rich juice, plasma CAT and GPx activity significantly increased from 4.92 (3.77–8.00) to 6.42 (4.33–8.24) nmol/min/mL ($p < 0.001$) and from 75.92 (69.10–90.18) to 85.22 (74.28–91.96) nmol/min/mL ($p < 0.05$), respectively. Again, CAT and GPx activity remained unchanged after placebo intake (Figure 4b,c).

The antioxidant capacity of plasma samples was estimated as TEAC comprising the antioxidative capacity of both lipophilic and hydrophilic compounds. Comparable to antioxidative enzyme activities in plasma, the median TEAC value significantly increased after ingestion of the anthocyanin-rich juice from 1.09 (1.02–1.19) to 1.21 (1.07–1.35) mmol/mL ($p < 0.001$). However, after placebo intake no significant change was observed.

(Figure 4d). In contrast to the observations for plasma antioxidative enzymes and capacity, no significant differences were found with respect to urine TPC after intake of both, placebo and juice (Figure 4e). Interestingly, heatmap analysis (Figure 4, upper panels) shows that GPx activity from vegetarians (volunteers 1–12) was higher than those of omnivores. In summary, these results show that consumption of an anthocyanin-rich juice for 28 days significantly improves the antioxidant status of healthy volunteers.

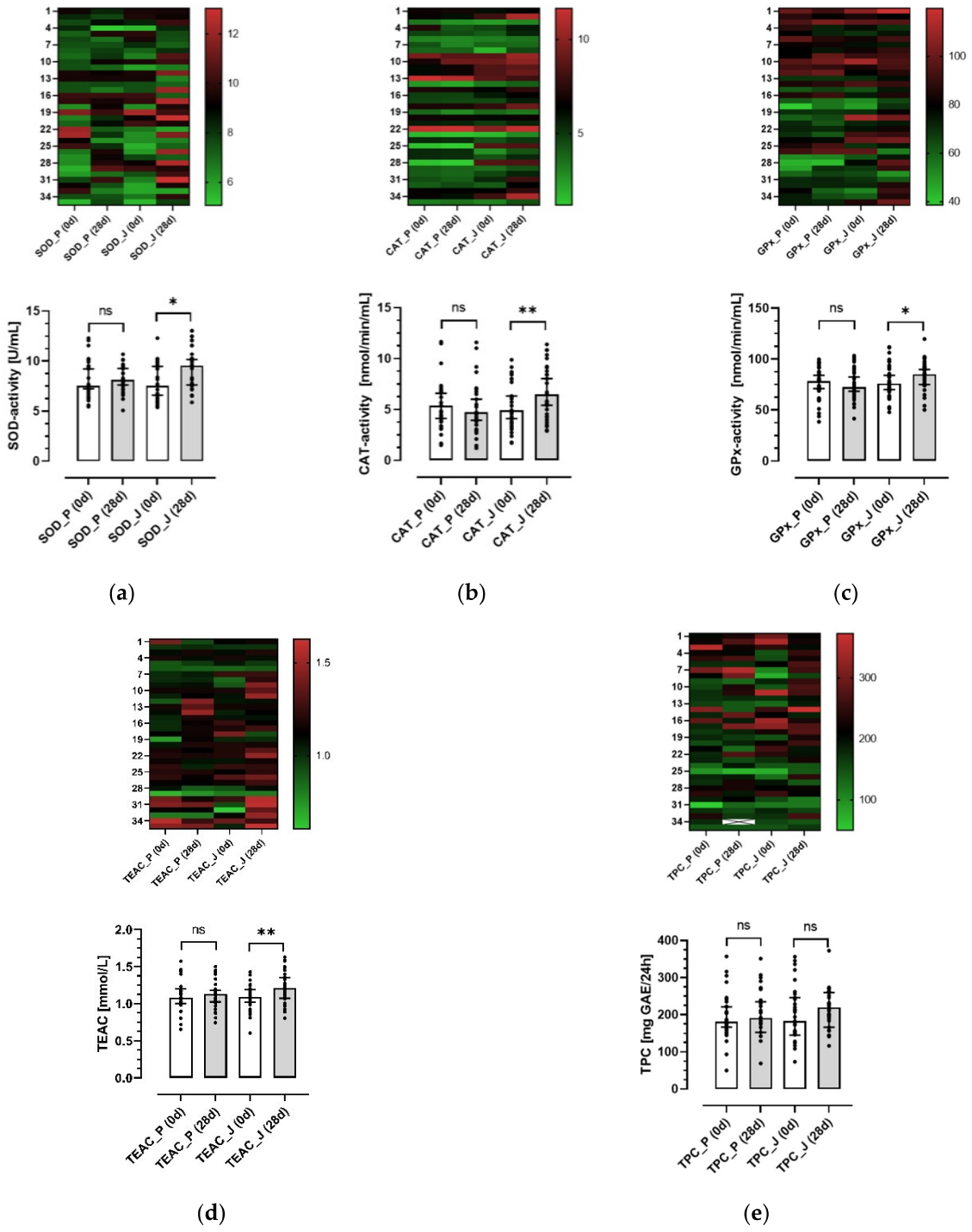


Figure 4. Effects of the anthocyanin-rich juice and the anthocyanin-depleted placebo on antioxidative parameters before and after intervention. Study participants consumed 0.33 L of the anthocyanin-depleted placebo (P) and anthocyanin-rich juice (J) over 28 days. Before (0 d) and after (28 d) intervention, blood samples were drawn and 24 h-urine samples were collected. Enzyme activities of SOD (U/mL) (**a**), CAT (nmol/min/mL) (**b**), GPx (nmol/min/mL) (**c**) as well as TEAC (mmol/mL) (**d**) were measured in plasma (n = 35). TPC (mg GAE/24 h) (**e**) was measured in urine (n = 34). Upper panel: Heatmap analyses showed single values for each participant. Lower panel: Values are presented as aligned dot blot bars with median and interquartile range (25th–75th). Significant differences were calculated with a mixed model with multiple comparison test (Šídák's) or ANOVA with multiple comparison test (Šídák's). Values after intervention were different with * $p < 0.05$, ** $p < 0.005$ to corresponding controls. ns, non significant.

3.5. Correlation between Parameters of Antioxidant Capacity and Migration

We further aimed to investigate, whether the increase in antioxidant status parameters in the plasma of healthy volunteers after 28-day consumption of the anthocyanin-rich juice was correlated with the significant decrease of HT-29 colon cancer cell migration that was observed in vitro (Figure 5). In fact, the higher the increase in SOD activity in plasma after consumption of the anthocyanin-rich juice (Figure 5a), the higher the anti-invasive effect was on HT-29 cell migration in vitro ($p < 0.05$). In contrast, no correlation was observed between other antioxidant parameters in plasma, indicating a possible link between plasma SOD activity and colon cancer metastasis.

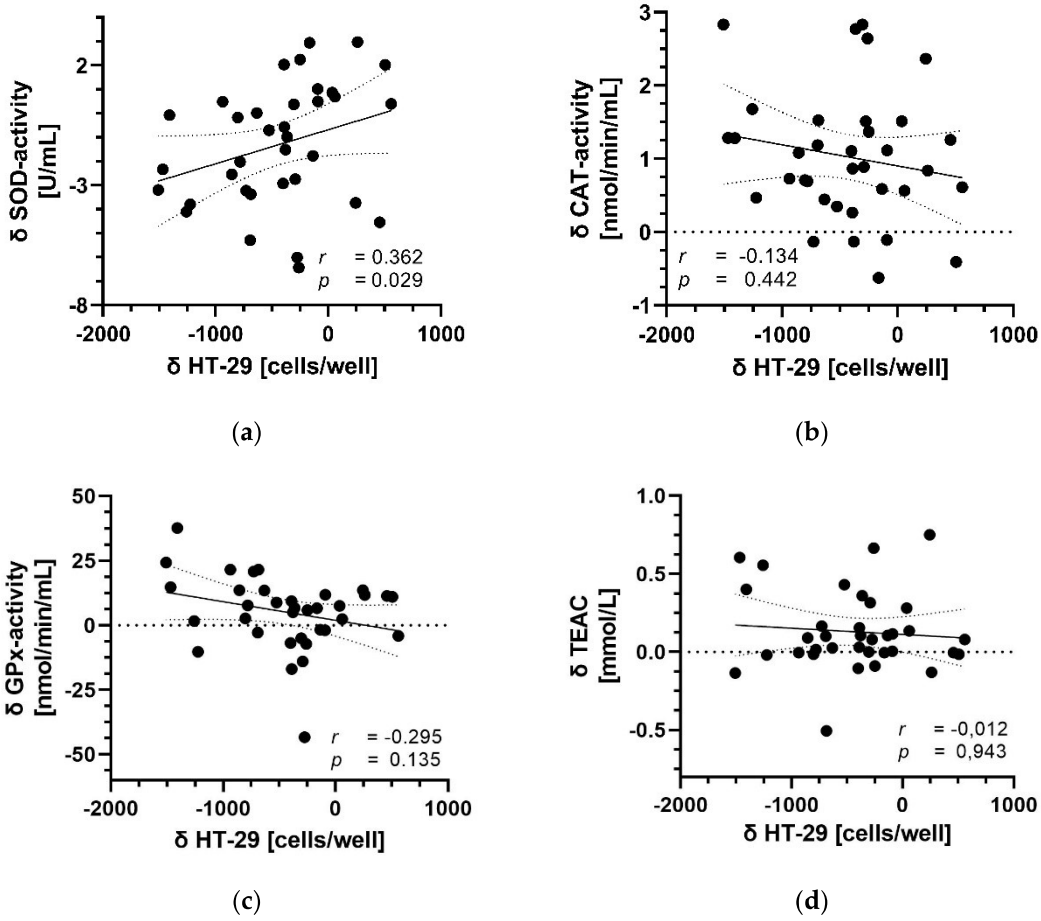


Figure 5. Scatter plots of antioxidant parameters and HT-29 migration. Correlation between HT-29 cell migration in vitro and SOD activity (**a**), CAT activity (**b**), GPx activity (**c**), and TEAC (**d**) in plasma. Correlation between the parameters were evaluated using Spearman correlation (r) with differences (δ) between values from day 28 and day 0 after anthocyanin-rich juice intake ($n = 35$).

4. Discussion

Considering that about every fourth colon cancer patient displays distant metastases after resection [52] and cancer mortality is mainly due to metastasis lesions [52,53], searching for new therapeutic agents to suppress cancer cell invasion and migration is crucial. However, data from human studies regarding the effects of anthocyanins on colon cancer progression are scarce and to date, it has not yet been clarified whether anthocyanins, particularly at physiological concentrations, have an impact on cancer cell migration in vivo. To overcome these limitations, the present study was designed to determine the anti-migratory effects of anthocyanins and their plasma metabolites on cell migration after long-term ingestion of an anthocyanin-rich grape/bilberry juice. Therefore, we combined a placebo-controlled, double-blinded, cross-over intervention study with in vitro migration

studies of two colon cancer cell lines, HT-29 and Caco-2. Healthy students daily consumed 0.33 L of an anthocyanin-rich juice in comparison to an anthocyanin-depleted placebo for 28 days. Blood samples were drawn before and after intervention. Subsequently, plasma samples were used to isolate PAM that were applied to a collagen-coated Boyden chamber for the measurement of colon cancer cell migration. To the best of our knowledge, this is the first study that showed that physiological concentrations of PAM significantly impair the migratory potential of HT-29 cells. In contrast, migration of Caco-2 cells was not affected. Although both cell lines were isolated from colon adenocarcinomas, they show differences in pheno- and genotypes, as well as in their grade of differentiation [54]. Hence, it is not surprising that undifferentiated HT-29 cells showed an obviously higher migratory potential compared with the more differentiated Caco-2 cells, which is underlined by the much higher number of migrated HT-29 cells in our experiments.

One of the most common and effective chemotherapeutic agents used for the treatment of CRC is 5-FU [50], although the benefit from 5-FU is often compromised by chemoresistance [49]. Therefore, it is vital to search for adjuvant agents and to investigate possible synergistic or even antagonistic effects of PAM on 5-FU treatment. Indeed, co-incubation of HT-29 cells with 5-FU and PAM further decreased cell migration compared with 5-FU treatment alone. Similarly, Li et al. recently reported that black raspberry anthocyanins significantly increased the anti-proliferative and anti-migratory effects of 5-FU and Celecoxib in colon cancer cell lines [55]. However, proliferation of Caco-2 cells was not affected by single treatment with black raspberry anthocyanins [55]. Accordingly, physiological concentrations of anthocyanins and their metabolites might possibly enhance the anti-migratory effect of 5-FU in Caco-2 cells. However, a possible synergistic effect of PAM and 5-FU from all volunteers was not investigated in the present study due to the limited sample material.

Metastasis is a multistep process that comprises cell migration from the primary tumor site, cell invasion, attachment to endothelial cells, extravasation, proliferation, and angiogenesis at the distal site [53,56]. Degradation of the extracellular matrix, especially collagen, mediated by proteolytic enzymes such as MMP-2 (gelatinase-A) and MMP-9 (gelatinase-B), is known to be the first step and plays a crucial role during cell migration and invasion [31,57]. While the anti-migratory and anti-invasive effects of anthocyanins have been reported for several cancer types, data regarding their impact on colon cancer cell migration are limited. The migration and invasion of HT-29 cells was significantly inhibited by an anthocyanin extract from *Vitis coignetiae Pulliat*, a member of the grape family, by suppressing MMP-2 and MMP-9 expression, which was likely due to the inhibition of NF-κB activation [25]. The inhibitory effects of anthocyanins on MMP-2 and MMP-9 expression were also shown in HCT-116 colon cancer cells [26]. Interestingly, Zhang et al., recently reported that black raspberry anthocyanins increased miR-24-1-5p expression in colon cancer cell lines, whereas miR-24-1-5p overexpression was associated with a significant decline of HCT-116 and Caco-2 migration [27]. However, these anti-invasive and anti-migratory effects were only shown at concentrations much higher than anthocyanin levels usually observed in plasma. Nevertheless, we have recently shown for the first time that physiological concentrations of anthocyanins and their metabolites isolated from the plasma of healthy volunteers are able to reduce tumor cell migration of the pancreatic cancer cell line PANC-1 in vitro [31]. This was accompanied by a significant reduction of ROS and decreased MMP-2 and MMP-9 levels, as well as NF-κB mRNA expression, suggesting that physiological concentrations of PAM may be adequate due to synergistic and additive effects. Although in the present study the underlying mechanisms remain unknown, it is most likely that inhibition of HT-29 cell migration is also attributed to suppression of the NF-κB pathway, as well as decreased expression of MMPs. In the present study the applied anthocyanin-rich juice was made from an eighty/twenty mixture of red grapes and bilberries with peonidin-3,5-O-diglucoside, malvidin-3,5-O-diglucoside, and peonidin-3-O-glucoside representing the major anthocyanins. It has been shown that peonidin-3-O-glucoside significantly inhibits MMP secretion and migration of lung cancer

cells [58]. In addition, peonidin has been reported to be the most potent anthocyanidin comparing the anti-migratory and anti-invasive capabilities of the five anthocyanidins cyanidin, malvidin, peonidin, petunidin, and delphinidin [59]. Peonidin significantly suppressed migration and invasion of highly invasive H1299 cells by about 20% even at a relatively low concentration (6.25 µM) [59]. Furthermore, synergistic anti-migratory and anti-invasive effects have been reported for a mixture comprising the five aglycons at equimolar concentrations [59]. However, this approach did not consider the impact of anthocyanin metabolites, which are much more abundant in plasma compared with native anthocyanins [38]. In addition, the bioavailability of native anthocyanins is low due to (1) their low stability and degradation at physiological pH values in the small intestine, (2) their metabolization through Phase II enzymes, and (3) their metabolism by the intestinal microbiota [60–62]. Regarding the pharmacokinetics of anthocyanins, native anthocyanins reach maximum plasma levels 30–60 min after ingestion [38]. In contrast to the ANTHONIA study [21], blood samples were drawn approximately 6 h after juice ingestion in the present study. At this time, Phase-II as well as microbiota-derived metabolites are present in the systemic circulation [63]. Therefore, the present study suggests that not only native anthocyanins but also their metabolites exert potent anti-migratory properties. However, we did not identify which anthocyanins and metabolites are responsible for the observed anti-migratory effects. Therefore, characterization of these compounds should be addressed in future studies.

Epidemiological studies indicate protective effects of dietary polyphenols against oxidation- and inflammation-related diseases such as cancer, cardiovascular diseases, diabetes mellitus, and neurodegenerative diseases [64,65]. It has been shown that the TAC of CRC patients is significantly lower compared with healthy controls [5,33]. Furthermore, CRC progression and metastasis are also associated with a significant decline in TAC, as well as lower CAT and GPx activities in serum and plasma, respectively [5,66]. Therefore, CRC patients may be more vulnerable to oxidative stress and the administering of anthocyanins might improve the antioxidant capacity, overall health, and outcome of CRC patients. Our results show that daily intake of an anthocyanin-rich juice for 28 days significantly improves plasma antioxidant capacity of healthy volunteers determined by enzymatic and non-enzymatic biomarkers. Plasma antioxidant capacity, measured as TEAC, was significantly higher after consumption of the anthocyanin-rich juice, whereas no change in TEAC was seen after ingestion of the anthocyanin-depleted placebo. Similarly, activities of the antioxidant enzymes SOD, CAT, and GPx were significantly increased after the juice, whereas the enzyme activities were not affected by the placebo. These results are in line with our recent findings from the ANTHONIA study [21]. Interestingly, GPx activity was not affected after consumption of an anthocyanin-rich grape/bilberry juice for 14 days [21]. Discrepancies in the results for GPx activity may be explained by different intervention times. In addition, there were slight differences between the grape/bilberry juices during the two studies. Both juices were made from an eighty/twenty mixture of red grapes and bilberries. Whereas in the ANTHONIA study the two grape varieties, *Dakapo* and *Accent*, were used, only the variety *Accent* was utilized in the present study, resulting in the different anthocyanin pattern of the juices. Thus, malvidin-3-O-glucoside and peonidin-3-O-glucoside were the major anthocyanin in the ANTHONIA study, whereas peonidin-3,5-O-diglucoside and malvidin-3,5-O-diglucoside represent the main anthocyanins in the present study. However, expression of the antioxidant enzymes SOD, CAT, and GPx is controlled by the redox sensitive transcription factor nuclear factor-erythroid 2 p45-related factor 2 (Nrf2) [67]. It is well known that anthocyanins, but also their gut-derived metabolites, are able to activate the Nrf2 pathway and consequently upregulate the defense against ROS and oxidative stress [68,69]. Compared with cells from the primary tumor site, metastatic cancer cells displayed higher ROS concentrations [6]. The ROS lowering effects of anthocyanins and their degradation products (gallic acid, syringic acid, protocatechuic acid, and phloroglucinol aldehyde) have been demonstrated in different colon cancer cell lines [67]. In addition, we have recently shown that physiological plasma concentrations

of PAM significantly reduce ROS generation in PANC-1 cells [31]. However, possible ROS lowering effects of PAM in HT-29 and Caco-2 cells have not been investigated in the present study. Recently, Yang et al., 2021 reported that blueberry anthocyanins significantly increased SOD activity in the lungs and livers of mice in vivo. In addition, in these organs reduced metastasis of breast cancer cells, which are known to be highly invasive, was observed [70], indicating a possible link between SOD activity and metastasis. However, to the best of our knowledge no study so far has investigated possible associations between the enzymatic and non-enzymatic antioxidant capacity in plasma and colon cancer cell migration. Interestingly, the higher the increase of SOD activity in plasma after 28 days intervention with the anthocyanin-rich juice, the higher the anti-invasive effect on HT-29 cell migration was in vitro. Although this correlation was weak, the impact of anthocyanins and their metabolites on tumor metastasis is much more complex in vivo. Besides direct effects on cancer cells, PAM may also influence other cell types such as immune cells [70] and consequently the microenvironment and migratory potential of tumor cells. To date, the role of anthocyanins during tumor migration of colon cancer patients is not fully understood and should be further investigated.

The present study has some limitations. First, participants were advised to adhere to a diet low in phenolics and anthocyanins during the run-in and intervention phase. These strong dietary limitations may not reflect a healthy and balanced diet. Furthermore, nutritional limitations were the reason for the high drop-out rate or termination of the study. Therefore, future studies should address whether the consumption of anthocyanin-rich beverages in addition to a normal diet also show health-promoting effects. Secondly, it remains to be shown which plasma anthocyanins and metabolites, as well as molecular mechanisms, are responsible for the anti-migratory effects. Finally, it could not be ruled out that the observed effects are in part mediated by other phenolic compounds also present in the anthocyanin-rich grape/bilberry juice.

5. Conclusions

To the best of our knowledge, we demonstrated for the first time that plasma-isolated anthocyanins and their metabolites significantly decrease migration of colon cancer cells in vitro. Furthermore, our data indicate that physiological concentrations of anthocyanins and their metabolites may enhance the efficacy of classical chemotherapeutic agents like 5-FU.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/antiox11071341/s1, Table S1: Foodstuffs that were restricted or not allowed during the run-in and 28-day intervention period; Table S2: Baseline characteristics and mean dietary intake of the study population during the two intervention periods (n = 34); Figure S1: Effects of PAM on HUVE cell viability.

Author Contributions: Principal investigator and conceptualization, S.K. and S.R.; migration studies, measurement of plasma antioxidant parameters and writing—original draft preparation, S.K. and I.B.; juice preparation and characterization, I.R. and F.W.; TPC analysis, H.M., T.M., R.G.-D. and C.A.-L.; writing—editing the manuscript, M.F. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the German Research Foundation (DFG, Deutsche Forschungsgemeinschaft) (grant # 1925/5-1) and further supported by CIBERFES, AC19/00096 funded by Instituto de Salud Carlos III and co-funded by European Regional Development Fund "A way to make Europe" and the award of the Generalitat de Catalunya's Agency AGAUR [2017SGR1546] and ICREA Academia 2018. T.M. would like to thank the "Juan de la Cierva" program from MINECO (IJCI-2017-32534). H.M. would like to thank the scholarship ajuts de personal investigador predoctoral en formació (APIF) from the University of Barcelona (Contract code: 404).

Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki, and approved by the Ethics Committee of the Justus Liebig University, Giessen (registration number: 13/10; date: 7 July 2017).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data are not publicly available due to European data protection regulations.

Acknowledgments: We are deeply grateful to all volunteers who participated in the study. The authors would also like to thank Cordula Becker, Katrin Koslowski, Sina Streichert, and Elvira Friedrich for their technical support in performing blood and urine analyses.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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Supplementary Material

Influence of plasma isolated anthocyanins and their metabolites on cancer cell migration (HT-29 and Caco-2): Results of the ATTACH study

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Table S1. Foodstuffs that were restricted or not allowed during the run-in and 28-day intervention period.

Foodstuffs allowed with **minor restrictions**[1]	Foodstuffs **not allowed**
Milk and milk products: milk, yoghurt and other milk products with fruit, nuts (no red fruit). **Vegetables**: yellow and green paprika, leek, spinach, cauliflower, broccoli, onions (no red onions). **Fruit**: bananas, pears, white grapes, apples (max. 1 piece/day), peaches, apricots (max. 1 piece/day), kiwis, pineapple. **Breakfast cereals**: cereals (no wholemeal/ without red fruits/ dark chocolate/ bran). **Fat, spices**: vegetable fat and oil, paprika spice, chili spice, cayenne pepper, curry spice. **Sweets**: ice cream with fruits, nuts (without red fruit / dark chocolate), marmalade/ jam/ jelly (without red fruit). **Allowed (per day):** 1 cup of tea, 1 cup of coffee, 1 glass of ale or glass of white wine (no red wine). Please note: fruit and vegetable, if possible, have to be peeled before consumption, especially, if consumed more portions a day, forgo red fruits, no consume external leaves of salad, no consume cereal products, listed spice and mixed spices use sparing.	**Fruit**: blueberries, blackberries, cranberries, strawberries, bilberries, raspberries, currants, lingonberries, cherries, red grapes, pomegranate, plums, watermelon. **Fruit containing foodstuffs**: red marmalade/ jellies, cake with red fruits, yoghurt with red fruits, ice cream with red fruits, desserts, muesli, muesli bars, cereals with wholemeal, dark chocolate, cocoa powder, gumdrop with fruit. **Vegetables**: purple potatoes, purple carrots, tomatoes, red and orange peppers, red cabbage, beetroot, radicchio, red radish, rhubarb, red and black pulses (kidney beans, red lentils), eggplant, pumpkin, red onions. **Other**: tomato sauce, puree, or paste, ketchup, red pesto. **Avoid** red wine, fruit and vegetable juice, fruit spritzer, smoothies, fruit tea, green tea, coffee, rooibos tea, alcoholic drinks (bear, wine, schnapps, and liqueur). Please note: anthocyanins may be food additives listed as number E 163.

[1]Foodstuffs were categorized according to their anthocyanin content based on data using the USDA database for the Flavonoid Content of Selected Foods (Release 3.3 (2018); http://www.ars.usda.gov/) or the database on polyphenol contents in food (Polyphenol-Explorer, Release 3.0 (http://www.phenol-explorer.eu/compounds)

Table S2. Baseline characteristics and mean dietary intake of the study population during the two intervention periods (n=34).

Dietary intake per day[1]	Intervention period 1	Intervention period 2
Age, *years*	24.4 ± 2.3	
Weight, *kg*	64.3 ± 11.7	63.2 ± 12.07
BMI, *kg/m²*	21.7 ± 2.6	21.3 ± 2.2
Dietary intake (3-day protocol)		
Energy, *kcal/d*	1698 ± 552	1855 ± 578
Fat, *g/d*	63.4 ± 17.9	64.4 ± 18.7
Carbohydrates, *g/d*	250.9 ± 63.2	245.8 ± 48.1
Dietary fibre, *g/d*	20.3 ± 5.3	19.4 ± 6.0
Protein, *g/d*	60.6 ± 16.4	54.7 ± 13.7
Retinol equivalents, *µg/d*	714 ± 263	682 ± 207
ß-carotene, *µg/d*	1,698 ± 1,520	1,387 ± 1,147
Vitamin E, *µg/d*	6,925 ± 6,630	7,201 ± 6,921
Vitamin C, *mg/d*	57.14 ± 34.2	63.88 ± 40.4

[1]Participants recorded their dietary intake over 3 days during each intervention period. Dietary records were analyzed using the DGE-PC professional software (Version 1.10.0.0) and values are expressed as means ± SD .

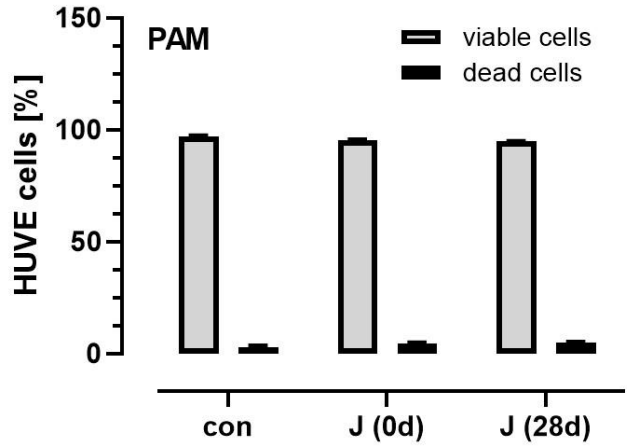


Figure S1. Effects of PAM on HUVE cell viability. Cells were seeded at a density of $1x10^5$ cells/mL in 24-well plates in complete medium with or without PAM from the anthocyanin-rich juice, which were isolated before (J (0d)) or after 28-days intervention (J (28d)) or with medium alone (con). After 36 h incubation, cells were washed twice with PBS, trypsinized and cell viability was measured using a Guava® Muse® Cell Analyzer. Data are expressed as bars [%] with standard deviation. Significant differences were calculated with ANOVA with multiple comparison test (n=2).

5.2.2. Publication 3. Plasma anthocyanins and their metabolites prevent in-vitro migration of pancreatic cancer cells, PANC-1, in a FAK- and NF-kB dependent manner: results from the ATTACH-study a randomized, controlled, crossover trial in healthy subjects. **Hamza Mostafa,** Inken Behrendt, Tomás Meroño, Raúl González-Domínguez, Mathias Fasshauer, Silvia Rudloff, Cristina Andres-Lacueva, Sabine Kuntz. *Biomedicine & Pharmacotherapy journal*. 158 (2023) 114076; https://doi.org/10.1016/j.biopha.2022.114076

Journal impact factor: 7.419

Journal quartile: Q1 (Category: Medicine, Research & Experimental, 30/139)

Abstract:

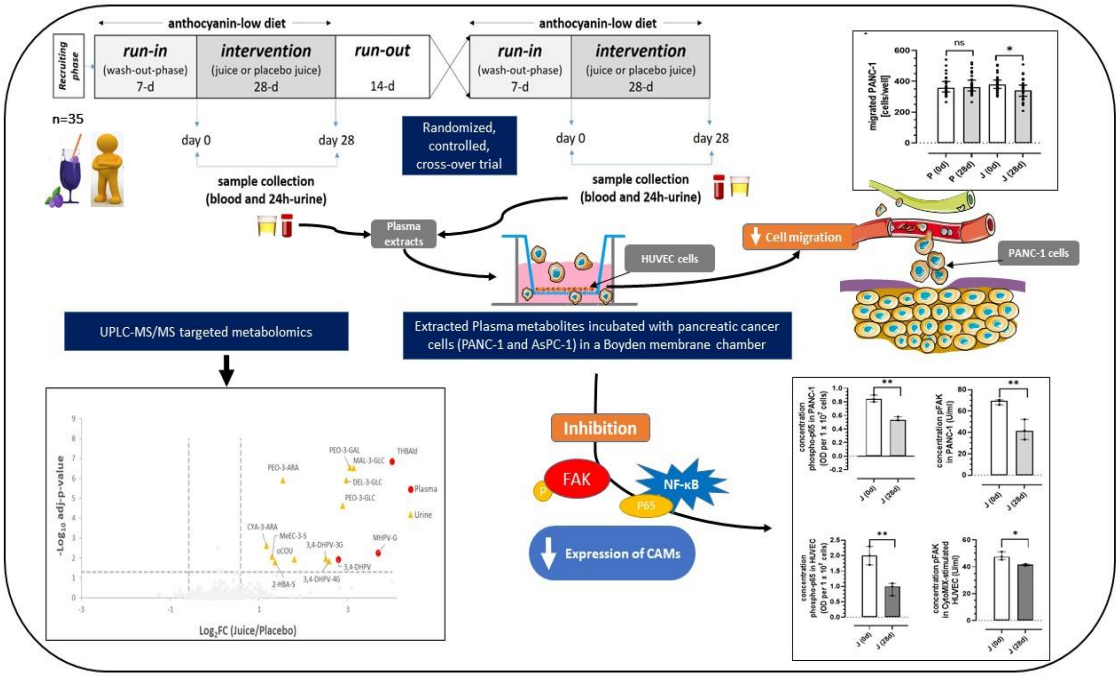
Pancreatic cancer is primarily considered to be a metastatic disease with a low 5-year survival rate. We aimed to detect if plasma-isolated anthocyanins and their metabolites (PAMs) modulate pancreatic cancer cells migration and to describe molecular targets of PAMs in this process. Plasma metabolites were isolated by solid-phase extraction before and after a 28-days intervention trial involving 35 healthy subjects comparing effects of a daily anthocyanin-rich juice intake vs. placebo. Plasma extracts were used for migration and mechanistic in vitro studies as well as for metabolomic analysis. Pancreatic PANC-1 and AsPC-1 were used for migration studies in a Boyden chamber co-cultured with endothelial cells. Expression of adhesion molecules on cancer and endothelial cells were determined by flow cytometry and NF-kB (nuclear factor-kappa B) p65 and focal adhesion kinase activation were measured by immunoassays. UHPLC-MS/MS metabolomics was done in plasma and urine samples. Plasma extracts isolated after the intake of the anthocyanin-rich juice significantly reduced PANC-1 migration, but not AsPC-1 migration. In PANC-1, and to a lower extent in endothelial cells, plasma extracts after juice intake decreased the expression of ß1- and ß4-integrins and intercellular adhesion molecule (ICAM)-1. Pooled plasma from volunteers with the highest inhibition of PANC-1 migration (n = 10) induced a reduction of NF-kBp65 and FAK-phosphorylation in cancer and in endothelial cells. Concerning metabolites, 14 were significantly altered by the juice intervention and PANC-1 migration was inversely associated with the increase of o-coumaric acid and peonidin-3-galactoside.

PAMs were associated with lower PANC-1 cell migration opening new strategies for metastatic pancreatic cancer treatment.

Resumen:

El cáncer de páncreas se considera principalmente una enfermedad metastásica con una baja tasa de supervivencia a 5 años. Nuestro objetivo fue detectar si las antocianinas aisladas del plasma y sus metabolitos (PAM) modulan la migración de las células del cáncer de páncreas y describir las dianas moleculares de las PAMs en este proceso. Se aislaron los metabolitos del plasma mediante extracción en fase sólida antes y después de un ensayo de intervención de 28 días que involucró a 35 sujetos sanos en los que se comparó los efectos de la ingesta diaria de zumo rico en antocianinas vs. placebo. Los extractos plasmáticos se utilizaron para estudios in vitro, así como para análisis metabolómicos. Los estudios de migración se realizaron en una cámara de Boyden co-cultivando células endoteliales (HUVEC) y células de cáncer de páncreas (PANC-1 y AsPC-1). La expresión de moléculas de adhesión en células cancerosas y endoteliales se determinó mediante citometría de flujo y se midió la activación de la quinasa de adhesión focal y NF-kB (factor nuclear kappa B) p65 mediante inmunoensayos. Se realizó metabolómica por UHPLC-MS/MS en muestras de plasma y orina. Los extractos plasmáticos aislados después de la ingesta de zumo rico en antocianinas redujeron significativamente la migración de PANC-1, pero no de AsPC-1. En PANC-1, y en menor medida en células endoteliales, los extractos plasmáticos después de la ingesta del zumo disminuyeron la expresión de ß1- y ß4-integrinas y la molécula de adhesión intercelular (ICAM)-1. El plasma agrupado de voluntarios con la mayor inhibición de la migración de PANC-1 (n = 10) indujo una reducción de la NF-kBp65 y la fosforilación de FAK en células cancerosas y endoteliales. En cuanto a los metabolitos, 14 se alteraron significativamente por la intervención con el zumo-rico en antocianinas y la migración de PANC-1 se asoció inversamente con el aumento del ácido o-cumárico y la peonidina-3-galactósido en orina. Las PAMs se asociaron con una menor migración de las células de PANC-1, lo que abre nuevas estrategias para el tratamiento del cáncer de páncreas metastásico.

Graphical abstract of the paper can be found next.



Biomedicine & Pharmacotherapy 158 (2023) 114076

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

journal homepage: www.elsevier.com/locate/biopha

Plasma anthocyanins and their metabolites reduce *in vitro* migration of pancreatic cancer cells, PANC-1, in a FAK- and NF-kB dependent manner: Results from the ATTACH-study a randomized, controlled, crossover trial in healthy subjects

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ARTICLE INFO

Keywords:
Anthocyanins
Metabolomics
Pancreatic cancer cells
Migration
Adhesion molecules

ABSTRACT

Pancreatic cancer is primarily considered to be a metastatic disease with a low 5-year survival rate. We aimed to detect if plasma-isolated anthocyanins and their metabolites (PAMs) modulate pancreatic cancer cells migration and to describe molecular targets of PAMs in this process. Plasma metabolites were isolated by solid-phase extraction before and after a 28-days intervention trial involving 35 healthy subjects comparing effects of a daily anthocyanin-rich juice intake *vs.* placebo. Plasma extracts were used for migration and mechanistic *in vitro* studies as well as for metabolomic analysis. Pancreatic PANC-1 and AsPC-1 were used for migration studies in a Boyden chamber co-cultured with endothelial cells. Expression of adhesion molecules on cancer and endothelial cells were determined by flow cytometry and NF-kB (nuclear factor-kappa B) p65 and focal adhesion kinase activation were measured by immunoassays. UHPLC-MS/MS metabolomics was done in plasma and urine samples. Plasma extracts isolated after the intake of the anthocyanin-rich juice significantly reduced PANC-1 migration, but not AsPC-1 migration. In PANC-1, and to a lower extent in endothelial cells, plasma extracts after juice intake decreased the expression of ß1- and ß4-integrins and intercellular adhesion molecule-1. Pooled plasma from volunteers with the highest inhibition of PANC-1 migration (n = 10) induced a reduction of NF-kB-p65 and FAK-phosphorylation in cancer and in endothelial cells. Concerning metabolites, 14 were significantly altered by juice intervention and PANC-1 migration was inversely associated with the increase of o-coumaric acid and peonidin-3-galactoside. PAMs were associated with lower PANC-1 cell migration opening new strategies for metastatic pancreatic cancer treatment.

1. Introduction

Pancreatic cancer is predicted to become the second leading cause of cancer death within the next decade in Western countries. It is commonly associated with poor prognosis and low overall five-year survival rate (5–7%) due to the early metastatic potential of pancreatic cancer cells [1]. Indeed, more than 80% of the patients are suffering from metastases or unresectable tumors at time of diagnosis [1].

Abbreviations: CAM, Cell Adhesion Molecules; ACN, Anthocyanins; HUVEC, Human Umbilical Vein Endothelial Cells; TEER, Transepithelial electrical resistance; FAK, Focal Adhesion Kinase; ROS, Reactive Oxygen Species.

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https://doi.org/10.1016/j.biopha.2022.114076

Received 6 October 2022; Received in revised form 25 November 2022; Accepted 2 December 2022

Metastasis is a multistep process in which cell adhesion molecules (CAMs), such as integrins and selectins play pivotal roles [2]. Malignant cells from the primary tumor infiltrate the surrounding tissue and enter the circulation by blood vessel intravasation and extravasation into the target tissue [2,3]. Alterations in the expression of several CAMs are known to modulate the migratory and invasive potential of cancer cells [4].

Strategies to improve the health outcomes in pancreatic cancer are challenging. Not only primary prevention strategies associated with a healthy lifestyle are discussed, but also secondary prevention strategies. Epidemiological studies revealed a potential benefit of diets high in fruits and vegetables [5–7]. A group of bioactive phytochemicals responsible for 'anti-cancer' effects are anthocyanins (ACNs). ACNs could account for up to 80% of the total polyphenolic content in berries [8]. However, ACNs bioavailability is low, and large amounts of dietary ACNs are fermented by gut microbiota [9,10]. As a result, several phenolic acids and other metabolites are generated [10,11]. Whether parent ACNs or their metabolites are responsible for these 'anti-cancer' effects is not fully understood. Recently, a screening of polyphenol metabolites on HCT116 3D-spheroids revealed that the ACN-related metabolite, 3′,4′-dihydroxyphenyl-γ-valerolactone (3,4-DHPV), reduced cancer cells spheroid integrity [12] underlining the role of gut microbiota fermentation of ACNs. Furthermore, a blueberry extract decreased CAM expression in several cancer cell lines and inhibited migration of breast and prostatic cancer cell lines (MDA-MB-231 and PC-3 cells), respectively [13]. Nonetheless, only few *in vitro* studies reported changes in CAM expression in cancer and endothelial cells in response to ACNs.

Previously, we showed that 60 min after a single ACN dose, plasma extracts from volunteers inhibited migration of the pancreatic cancer cell line PANC-1 *in vitro* [14]. Furthermore, we observed a reduction of migration of the colon cancer cell line HT-29 after a daily ingestion of ACNs over 28 days [15]. Therefore, our aims were to investigate: 1) whether plasma metabolites, isolated after a 28-day intervention, would reduce migration of two pancreatic cancer cell lines (PANC-1 and AsPC-1); 2) whether expression of adhesion molecules on cancer and endothelial cells were influenced by plasma ACN metabolites; 3) which molecular mechanisms were involved; and 4) which metabolites in plasma and urine were altered during a long-term ACN intake and how they associate with the inhibitory effects on migration.

2. Materials and methods

All details on materials are given in the Supplementary Table 1.

2.1. Study design and subjects

Details on the ATTACH study (Anthocyanins Target Tumor cell Adhesion—Cancer vs. Endothelial Cell (HUVEC)) have been published previously [15]. Briefly, this was a randomised, double-blind, placebo-controlled, cross-over, 28-days intervention comparing the effects of an ACN-rich juice (J) (330 ml/day, 942 mg/L of ACNs and 2622 mg/L of total polyphenols) and an ACN-depleted (330 ml/day, 6.3 mg/L of ACNs and 115 mg/L of total polyphenols) placebo juice (P), with a 2-week wash-out and 1-week run-in period. Thirty-five young, healthy volunteers participated in the intervention. Sample size was calculated based on the results of our previous published migration study [14] with ß- and α-error of 0.8 and 0.05 and a drop-out rate of 20%. CONSORT flowchart diagram is shown in Supplementary Figure 1. Participants were randomly assigned (random-table). The study protocol was approved by the local ethic committee in Gießen (Germany) (registration number 13/10) and performed according to the guidelines laid down in the Declaration of Helsinki. Written informed consent was obtained from all participants and the trial is registered at DRKS (*Deutsche Register Klinischer Studien;* DRKS00014767). The present manuscript is reported following the CONSORT checklist Supplementary Table 2.

2.2. Blood and urine sample preparation

Blood and 24 h-urine samples were collected before (day 0) and at the end (day 28) of the two intervention periods. Blood was taken 6 h after beverages intake of day 28 into EDTA-tubes and centrifuged to separate plasma. Acidified urine samples were then stored at − 80 °C until assayed. Extraction of plasma metabolites for cell culture assays and plasma and urine sample preparation for metabolomics analysis are described in the Supplementary Data (S1 and S2).

2.3. Cell culture and functional assays

Details on cancer cell lines and endothelial cells are given in the Supplementary Data (S3 and S4).

2.4. Outcomes

2.4.1. Primary outcome

2.4.1.1. Cell migration of pancreatic cancer cells in-vitro. Tumor cell migration was assessed in a Boyden membrane chamber with the use of the CytoSelect 24-well Cell Migration Assay as described previously [14,15]. The 24-well feeder chamber (diameter of the chamber 6.5 mm; pore size 8 μm) tray was coated with 50 μL of 0.1% fibronectin and aspirated until dryness. Thereafter, HUVECs (5×10^4/ml) were seeded onto the fibronectin-coated inserts and allow to grow confluent. Two-days post-confluence, transepithelial electrical resistance (TEER) was determined before the experiments by using a Millicell® ERS volt-ohmmeter. A TEER value $\geq$ 250 Ω per cm^2 was used as an indicator for an intact endothelial layer suitable to be used for functional studies. Pancreatic cancer cells (1×10^5/ml) were seeded in Endothelial growth medium II supplemented with 2.5% fetal calf serum (FCS) containing diluted plasma extracts of the participants, whereas Endothelial growth medium II supplemented with 12.5% FCS was added to the lower basal chamber. The cells were incubated in the feeder tray for 36 h at 37 °C, and cells on the lower side were then detached from the membrane using a cell detachment solution and afterwards lysed with fluorescent dye-containing buffer. The extent of migration was assessed by the intensity of the fluorescence signal with a microplate fluorescence reader. The number of migrated cells was determined according to a calibration curve (0–7500 cells). The results are expressed as medians and IQR (25th-75th).

2.4.2. Secondary outcome

2.4.2.1. Analysis of CAMs by flow cytometry analysis. Basal and `CytoMIX´-stimulated expression of surface marker such as cell adhesion molecules (CAMs) and Vascular endothelial growth factor receptor (VEGF-R) were analyzed under pre-confluent (PANC-1 and AsPC-1) or post-confluent (HUVECs) conditions. Cells were washed twice with phosphate buffered saline (PBS) and detached with TrypLE™ Express-solution for cancer cells or accutase-solution (0.15 ml/cm^2) for endothelial cells. After the detachment, cells were centrifugated (220xg for 3 min) and supernatant was decanted. Pellet was washed twice with PBS, resuspended in cold 100 μL MACS Running Buffer (pH 7.2) for staining procedures. Results are expressed as means ± SD or median with IQR (25th-75th). More details about staining procedures are given in the Supplementary data (S5).

2.4.2.2. Cytokine quantification, detection of NF-kB p65, FAK (focal adhesion kinase) activation and reactive oxygen species (ROS) determination. Commercially available ELISAs were used to determine cytokines, activation of NF-kB p65 and FAK in the supernatants of cells and were done by the manufacturer's instructions. ROS determination and sample preparation have been described previously (14). The results are

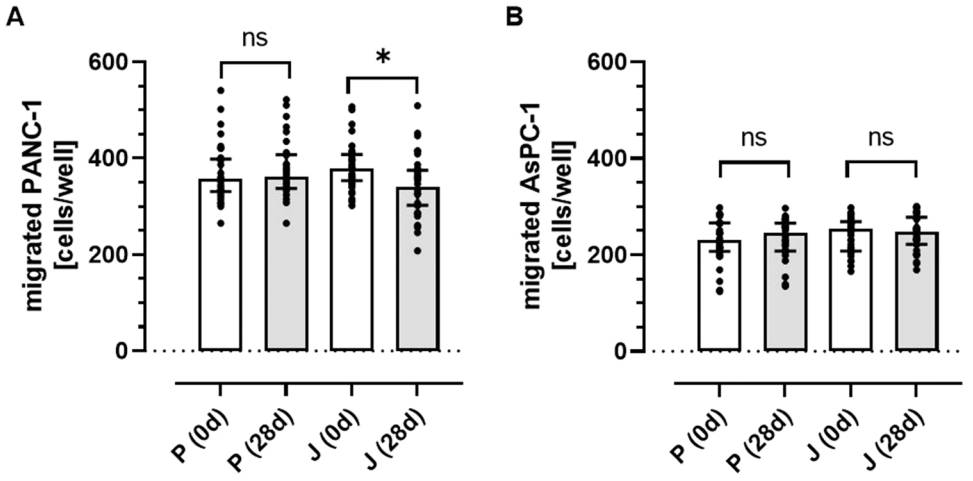

Fig. 1. Migration of PANC-1 and AsPC-1 in a Boyden chamber co-cultured with HUVECs representing endothelial cells *in vitro*. PANC-1 (A) and AsPC-1 (B) were exposed to plasma extracts from the placebo (P) and juice (J) intake before (0d) and after (28d) intervention. Migration across the endothelial layer into the lower chamber was measured after 36 h. Migration was measured fluorometrically in a Boyden chamber as described in Materials and Methods Section 2.4.1. Values are expressed as aligned dot blot with median and interquartile range (25th-75th). Significant differences were calculated with repeated measurements One-way ANOVA and values with * were different with *p-value* < 0.05 to corresponding controls (n = 34).

expressed as mean ± SD and more details are given in the Supplementary data (S6-S8).

2.4.3. Exploratory outcome

2.4.3.1. Targeted metabolomics UHPLC-MS/MS analysis. The UHPLC-MS/MS analysis has been explained previously [16]. Briefly, the analysis of plasma and urine was performed by using the 1290 Infinity UHPLC system coupled to a QTRAP 6500 mass spectrometer equipped with Ion Drive Turbo V ion source. Luna Omega Polar C18 column, 100 mm × 2.1 mm (i.d. 1.6 μm) with a porous polar C18 security guard cartridge were used to perform the chromatographic separation. More details are described in the Supplementary data (S9 and 10).

2.5. Statistical analysis

2.5.1. Cell culture data analyses

Data from the volunteers who completed all phases of the study were analyzed (n = 35). The outcome measures were prospectively designated as the differences in migration of PANC-1 and AsPC-1 *in vitro* (primary outcome) and mechanistic parameters (secondary outcome) of placebo and juice treatment before and after intervention. Before-treatment versus after-treatment data within groups were analyzed using a repeated measures one-way ANOVA with Šídák's post hoc test. The normality of continuous variables was assessed using Kolmogorov-Smirnov normality test. Asterisks are used in the figures to denote *p* values < 0.05, which were considered significant. GraphPad Prism 9 (Version 9.3.1.) was used for data analyses.

2.5.2. Metabolomics data analyses

Pre-processing of metabolomics data is described in the Supplementary Data (S10). To assess the effects of the juice intervention, we used a linear mixed model (LMM) including treatment (juice/placebo), age, and sex as fixed effects and subject as random effect. *P*-values were adjusted for multiple comparisons using the Benjamini-Hochberg false discovery rate (FDR) and results were integrated in a volcano plot. We selected as significant those metabolites with a $\log_2 FC > |0.584|$ and an FDR-adjusted *p*-value < 0.05. Associations between plasma anthocyanin metabolites and migration experiments and adhesion molecules expression were tested using linear mixed models adjusted for age, sex and treatment as fixed effects, and subject as random effect. R statistical software version 4.1.3 was used for metabolomics statistical analyses.

3. Results & discussion

Thirty-five volunteers (female n = 27 and male n = 8), with a mean (± SD) age of 24.4 (± 2.3) years old, an initial body weight of 64 ± 18 kg and a BMI of 21.7 ± 2.6 kg/m^2, completed the protocol while one

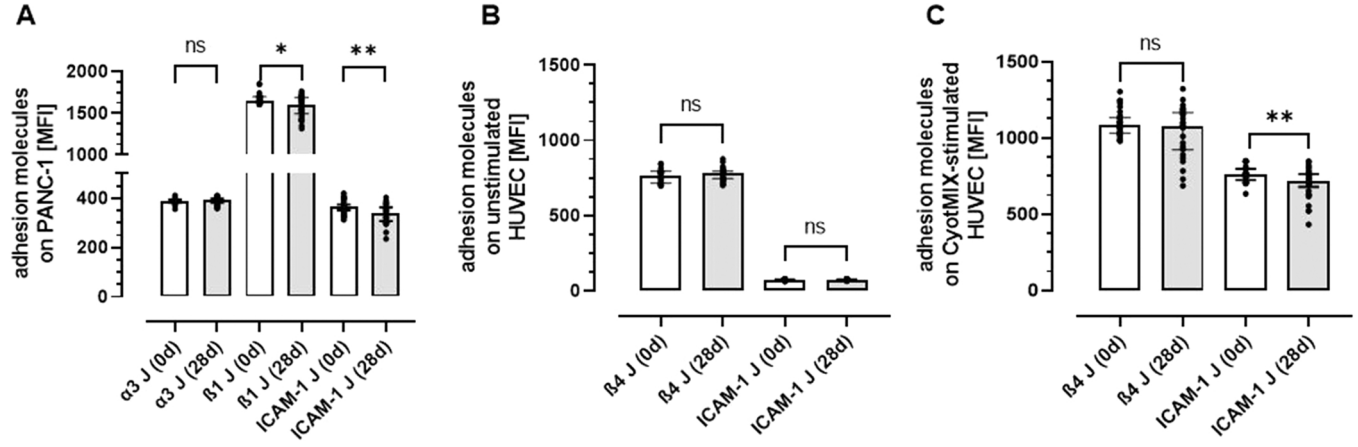

Fig. 2. Expression of CAMs on cancer cells and endothelial cells after incubation with plasma extracts from ACN-rich juice. Expression of adhesion molecules on PANC-1 (A) as well as on non-stimulated and CytoMIX-stimulated HUVECs (B and C) after incubation with plasma extracts from volunteers ingested the ACN-rich juice (J) over 28 days. CAMs were measured fluorometrically by flow cytometry as described in the Materials and Method Section 2.4.2. Values are expressed as median of main fluorescence intensity (MFI) with interquartile range (25th–75th). Significant differences were calculated with repeated measurements One-way ANOVA and values with * , * * and * ** were different with *p-value* < 0.05, *p-value* < 0.01 and *p-value* < 0.001 to corresponding controls (n = 34).

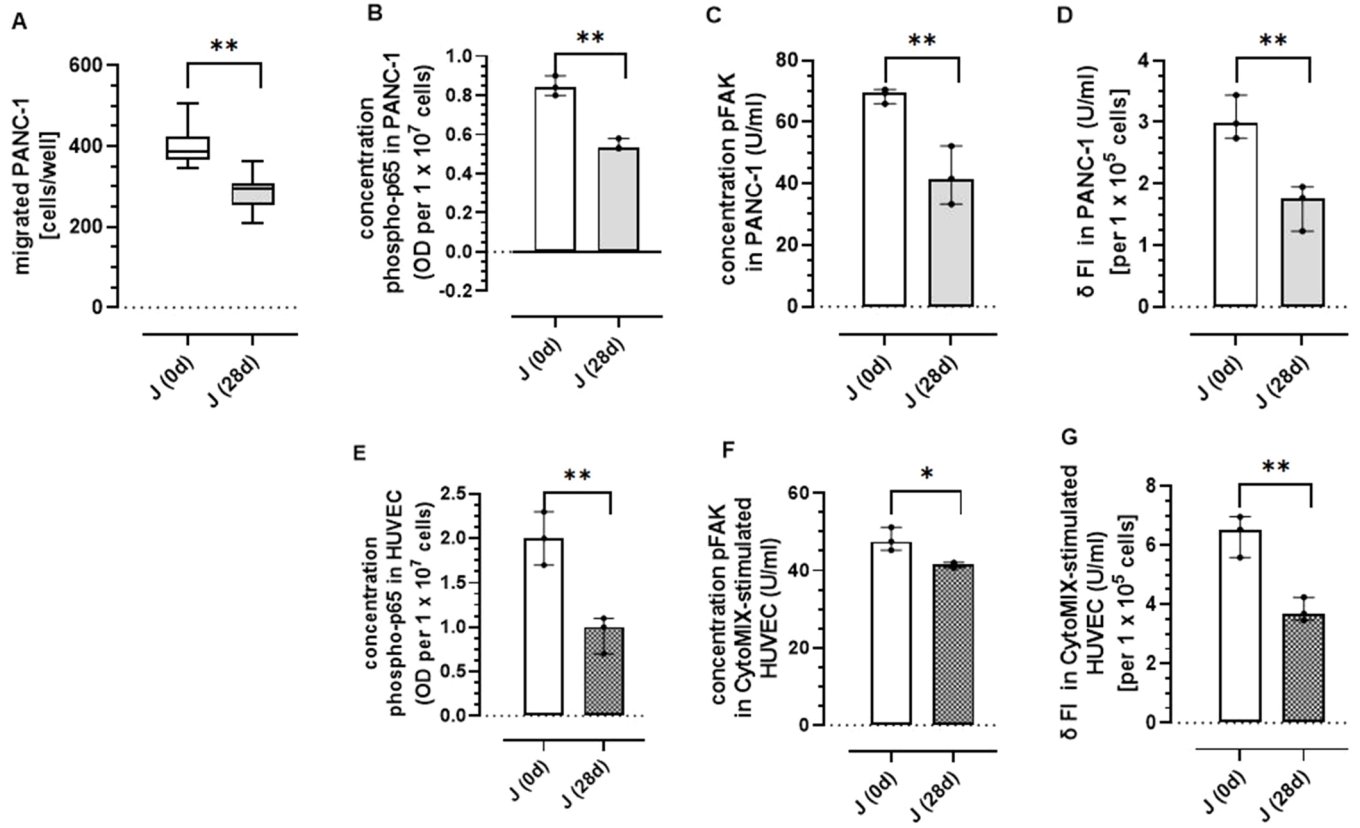

Fig. 3. Molecular Targets of plasma extracts previous and after juice intervention in PANC-1 and HUVECs. (A). PANC-1 migration across the endothelial layer into the lower chamber of co-culture model were measured after 36 h with pooled plasma metabolites of the 10 samples with the highest percentage of migration reduction). (B-G) PANC-1 and ĆytoMIX´-stimulated HUVECs were seeded onto 6-well plates and incubated with pooled PAMs for 36 h. Thereafter, cells were lysed and phosphorylation of p65 (B, E) and FAK (C, F) were measured colorimetrically by ELISA as described in the Materials and Method Section 2.4.2. (D, G) ROS generation was measured after 36-h exposure to PAMs from ACN-rich juice (J) before (d0) and after 28-d (d28) intervention. Thereafter, cells were washed with PBS and incubated with 5 μmol/L H_2DCFDA for 30 min at 37 °C. Fluorescence intensity was measured (Ex/Em 485/535) as described in Materials and Methods Section 2.4.2. Values are expressed as means with standard deviation. Significant differences were calculated with t-test and values with * * were different with *p-value* < 0.01 compared to corresponding controls (n = 3).

subject had an incomplete urine sample collection (blood n = 35; urine n = 34).

3.1. *Primary and secondary outcome: cancer cell migration, expression of adhesion molecules and mechanism*

As shown in Fig. 1, plasma extracts had different effects on cancer cell migration *in vitro* depending on the cancer cell line. In PANC-1, extracted-plasma metabolites after the ACN-rich juice reduced cell migration significantly in comparison to plasma extracts after placebo (Fig. 1A). On the other hand, no reduction was observed for the migration of AsPC-1 (Fig. 1B). As viability of PANC-1 and AsPC-1 was not affected by incubation with plasma extracts under the experimental conditions (data not shown), one reason for these differences could be the influence of plasma extracts on expression of surface molecules and secretion of cytokines that stimulate endothelial cells (Supplementary data S11 and S12). Briefly, in comparison to AsPC-1, PANC-1 expressed higher levels of α3-, α4-, ß1- and ß2-integrin, E-selectin, ICAM-1 and VEGF-R. However, only ß1-integrin and ICAM-1 expression were significantly reduced on PANC-1 by plasma metabolites extracted after juice intake (Fig. 2A). Furthermore, PANC-1 showed a higher secretion of cytokines, such as TNF-α, IL-1ß, and VEGF compared to AsPC-1, indicating a higher potential for activating CAMs on endothelial cells (Supplementary data S4). Higher expression of CAMs and cytokines secretion may be responsible for higher interaction with endothelial cells and thus migration of PANC-1 across the endothelial layer. To investigate the effect of plasma extracts after juice intake on CAMs of HUVECs, cells were stimulated for 36 h with a so-called ĆytoMIX´ (TNF-α, IL-1ß and VEGF), which were secreted by cancer cells. As shown in Fig. 2B and Fig. 2C, CytoMIX-stimulation resulted in increased expression of ß4-integrin and ICAM-1 in comparison to non-stimulated HUVECs, whereas plasma extracts after juice intake significantly reduced only CytoMIX-stimulated ICAM-1 expression.

Expression of CAMs on tumor cells and the expression of receptors on endothelial cells are important steps in the multistep-cascade of migration and metastasis [17]. It has previously been shown that ACNs were able to reduce cancer cell migration by inhibiting phosphorylation of signalling proteins [18,19]; however, we acknowledge that 311 mg/day of ACNs would be difficult to achieve within a diet. However, compared to other studies we used ACN and their metabolites isolated from plasma samples. Most of the previous studies examined the relation between ACN-rich diet and anti-cancer activities using food frequency questionnaires, or after extracting the ACN-related phenolic metabolites from a prepared stock solution directly used in cancer cells [20–24]. However, this result support studies showing that cancer chemoprevention combined with bioactive phytochemicals could be associated with less toxicity and improved efficacy [25–27].

To investigate the mechanisms behind the observed anti-migratory effects on PANC-1, we pooled the plasma extracts from the volunteers with the highest percentage of migration reduction (n = 10). NF-kB p65 and FAK phosphorylation as well as ROS generation were analyzed after incubation with this pool of extracted plasma samples. The redox-sensitive NF-kB and FAK pathways are associated with the regulation of adhesion molecules. As shown in Fig. 3A, incubation of cells with

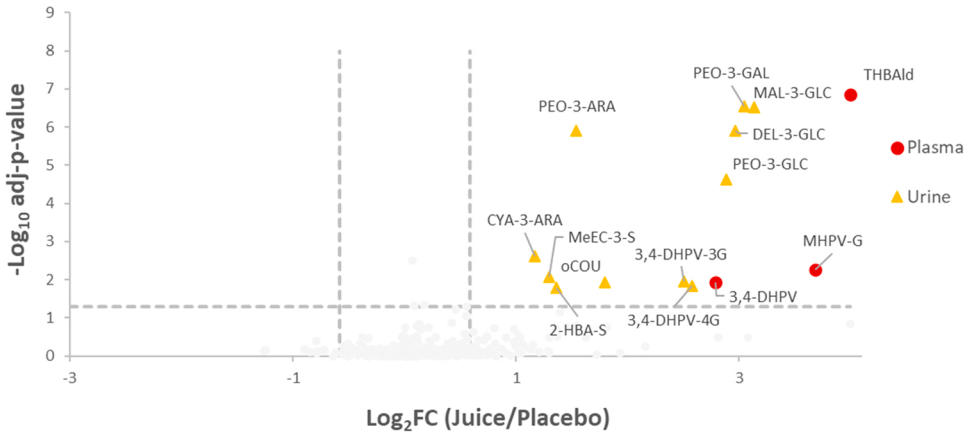

Fig. 4. Volcano plot of log_2FC vs log_{10} FDR-adjusted p-value, showing the metabolites that are significantly associated with ACNs intake in plasma, and urine. The FC value of each metabolite corresponds to the difference between log_2FCpost/pre during ACN-rich juice and log_2FCpost/pre during placebo intervention. To determine the effect of the ACN-rich juice, LMM was used with treatment (juice/placebo), sex and age as fixed factors and subject ID as random factor. *p*-values were calculated and adjusted using FDR (plasma n = 35 and urine n = 34). THBAld: 2,4,6-trihydroxybenzaldehyde; MHPV-G: 4′-hydroxy-3′-methoxyphenyl-γ-valerolactone glucuronide; 3,4-DHPV: 3′,4′-dihydroxyphenyl-γ-valerolactone; oCOU: o-coumaric acid; MeEC-3-S: 3′-methyl(epi)catechin sulphate; 2-HBA-S: 2-hydroxybenzoic acid sulphate; 3,4-DHPV-3 G: 3′,4′-dihydroxyphenyl-γ-valerolactone 3′-glucuronide; 3,4-DHPV-4 G: 3′,4′-dihydroxyphenyl-γ-valerolactone 4′-glucuronide; PEO-3-GAL: peonidin-3-galactoside; PEO-3-GLC: peonidin-3-glucoside; PEO-3-ARA: peonidin-3-arabinoside; CYA-3-ARA: cyanidin-3-arabinoside; MAL-3-GLC: Malvidin-3-glucoside; DEL-3-GLC: delphinidin-3-glucoside; PET-3-GLC: Petunidin-3-glucoside).

pooled plasma extracts after juice intervention over 36 h significantly reduced cell migration. Concomitantly, incubation with plasma extracts after juice intervention significantly blocked phosphorylation of p65 and FAK in PANC-1 and also in CytoMIX-stimulated HUVECs (Fig. 3B-C and E-F) and reduced ROS levels in PANC-1 and stimulated HUVECs (Fig. 3D and G). FAK and NF-kB p65 were associated with increased cancer cell migration and cancer metastasis through higher expression of α- and ß-integrins [28,29]. Therefore, reduction of intracellular cell signalling pathways by plasma ACN metabolites opens a new window concerning cancer preventive strategies.

3.2. *Exploratory outcomes: metabolomics analyses*

From plasma and urine metabolome, 14 out of 902 metabolites (379 in plasma and 523 in urine) were significantly associated with ACN-rich juice intake as shown in Fig. 4. Among these, 3 plasma metabolites resulting from ACN-gut microbiota metabolism were found to be increased: 2,4,6-trihydroxybenzaldehyde (THBAld), 4′-hydroxy-3′-methoxyphenyl-γ-valerolactone glucuronide (MHPV-G) and 3′,4′-dihydroxyphenyl-γ-valerolactone (3,4-DHPV). In urine, 11 metabolites increased after the ACN-rich juice intervention; from these, 6 metabolites were parent ACNs (peonidin-3-galactoside, peonidin-3-glucoside, peonidin-3-arabinoside, cyaniding-3-arabinoside, malvidin-3-glucoside and delphinidin-3-glucoside), while the other 5 metabolites were gut microbial phenolic metabolites (o-coumaric acid (oCOU), 3′-methyl-epicatechin sulphate (MeEC-3-S), 2-hydroxybenzoic acid-sulphate, 3,4-DHPV-3-glucuronide, and 3,4-DHPV-4-glucuronide). In particular, THBAld, 3,4-DHPV and MHPV-G had been shown to have anti-cancer activities, but at supra-physiological doses [22–24,30]. However, we did not observe any association between the increment of plasma THBAld, 3,4-DHPV and MHPV-G and the reduction in PANC-1 migration. Because ACNs can be rapidly absorbed in the stomach, reach the blood and disappear from the circulation within few hours [31,32], it was expected that parent ACNs were only significantly altered in urine and not in plasma. Associations between PANC-1 migration as well as CAM expression of PANC-1 and of stimulated HUVECs with plasma and urine metabolites altered by ACN-rich juice are shown in Supplementary Figure 6. The increment of peonidin and oCOU in urine were inversely associated with PANC-1 migration. Similarly, ICAM-1 expression in PANC-1 was inversely associated with the increase in urinary excretion of MeEC-3-S.

Limitations of our study are the use of extracted plasma from young and healthy volunteers on cancer cell lines *in vitro*. Due to the intense metabolization and fermentation of ACNs, which may vary according to age, sex, and lifestyle, we cannot ascertain that these anti-migratory effects would be observed in patients with cancer or if there would be any interaction with classical chemotherapeutics.

In conclusion, the study showed for the first time that ACN and metabolites isolated from plasma after a long-term ACN-rich juice intervention reduced the migration and expression of CAMs in PANC-1 cancer cells *in vitro* through activation of FAK- and NF-kB-pathways as well as the reduction of ROS. These results are promising and could open a window to investigate interactions of ACNs with classical cancer prevention strategies.

CRediT authorship contribution statement

HM, TM, RGD and CAL: Metabolome analyses. **IB, MF, SR and SK:** conceptualization of the study, migration studies and mechanistic analyses. TM, IB and SK performed the statistical analyses. HM, TM, and IB wrote the first draft of the manuscript. All the authors reviewed and approved the final version of the manuscript.

Conflict of interest statement

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgment

We are deeply grateful to all volunteers who participated in the study. The authors would like to thank Cordula Becker, Katrin Koslowski and Elvira Friedrich for their excellent technical support. The study was funded by the DFG (grant # 1925/5–1) and further supported by CIBERFES, AC19/00096 funded by Instituto de Salud Carlos III and co-funded by European Regional Development Fund "A way to make Europe" and the award of the Generalitat de Catalunya's Agency AGAUR [2017SGR1546] and ICREA Academia 2018. HM would like to thank the scholarship ajuts de personal investigador predoctoral en formació (APIF) from the University of Barcelona.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2022.114076.

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Plasma anthocyanins and their metabolites reduce *in vitro* migration of pancreatic cancer cells, PANC-1, in a FAK- and NF-kB dependent manner: results from the ATTACH-study a randomized, controlled, crossover trial in healthy subjects.

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Supplementary methods

S1. Solid phase extraction (SPE) of plasma samples for cell culture assays. Plasma extraction of anthocyanins and their metabolites was based on the method described recently [Behrendt et al 2022, Kuntz S et al 2017]. Briefly, each aliquot (5 mL of plasma acidified with 150 µl of 50% aqueous formic acid (LGC Promochem, Wesel, Germany)) was loaded onto an Oasis-HLB (5 mL/200 mg) SPE cartridge (Waters, Inc., Eschborn, Germany), preconditioned with 5 mL of methanol (Thermo Fischer Scientific, Langenselbold, Germany) and 1% formic acid, followed by 5 mL of acidified water (1% formic acid). The cartridge was then washed with 5 mL of acidified water, after which anthocyanins and their metabolites were eluted with 5 mL of acidified methanol. Afterwards, N_2 gas was applied for 2h to dry the extract which was then stored at -80ºC until further analysis. For cell culture studies, SPE-isolated metabolites from 1 mL plasma were diluted in 1 mL DMEM (Dulbecco's modified Eagle's medium), 1 mL RPMI 1640 (Roswell Park Memorial Institute) media or 1 ml Endothelial growth medium II (Promocell GmbH, Germany), adjusted to pH 7.4, sterile filtered (Micro-Spin Eppendorf filter, pore size 0.2 µm; Eppendorf, Eugendorf, Germany) and immediately used for cell culture studies.

S2. Plasma and urine sample preparation. Plasma and urine sample preparation including all chemicals and standards used in this study have been described previously [González-Domínguez R et al 2020]. Plasma samples were prepared based on the protein precipitation approach. Briefly, the

precipitation solution was made of cold acetonitrile containing 1.5 M formic acid and 10 mM ammonium formate. The reconstitution solution was water:acetonitrile (80:20, v/v) containing 0.1% formic acid and 100 µg/L of a mixture of 13 internal standards (ISs). Using a Sirocco protein precipitation plate, 100 µl of plasma were added and mixed with 400 µl of the protein precipitation solution. The plate was then kept in a -20ºC freezer for 10 min to promote the protein precipitation. N_2 pressure was applied and the extract was collected in a 96-well collection plate and transferred to the speed vacuum concentrator until dryness. Finally, the samples were reconstituted with 100 µl of the reconstitution solution containing the ISs mix and transferred to Agilent well plates for further analysis. Urine samples were treated in two different procedures. Urine samples were taken from the -80ºC freezer and kept to be thawed and centrifuged for 10 min at 4ºC at 10,000xg. The 13 ISs mix mentioned above was used as ISs at a concentration of 100 µg/L. The first treatment was urine dilution, i.e., a 50 µl aliquot of the centrifuged urine was diluted with 200 µl of ultrapure water containing 0.1% formic acid (1:4 v/v) and the ISs- solution, and then transferred to 96-well plates for further analysis. The second treatment was a reversed solid phase extraction (SPE) using Oasis HLB plates. Conditioning of the plates was done by adding 1 ml of methanol and 1 ml of the equilibration solution consisting of water with 0.1% formic acid and 10 mM of ammonium formate. Then, 1 ml of urine containing 20 µl of 2% orthophosphoric acid and the set of the 13 ISs mix was loaded, followed by 1 ml of the equilibration solution. Then, 1.5 ml of the elution solution were added consisting of methanol with 0.1% formic acid and 10 mM ammonium formate. Finally, a speed vacuum concentrator (Gyrozen®, scanspeed 32) was used to dry the extracts which were then reconstituted with 100 µl of the previously mentioned reconstitution solution and transferred to Agilent well plates for further analysis.

S3. Cancer Cell lines. The two human pancreatic carcinoma cell lines, PANC-1 and AsPC-1 were purchased from American Type Culture Collection (ATCC) and cultured following the recommendations of ATCC (http://www.lgcstandards-atcc.org/). PANC-1 is an epithelioid carcinoma cell line derived from ductal cell origin of the human pancreas from a 56-year-old Caucasian male (3). PANC-1 cells were sub-cultured twice a week in DMEM and supplemented with 5 mmol/L L-glutamine, 1 mmol/L sodium pyruvate and 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere containing 5% CO_2. AsPC-1, a human pancreatic adenocarcinoma cell line derived from metastatic sites (ascites) of a 62-year-old Caucasian female were grown in RPMI 1640 medium and supplemented as described above for PANC-1. After reaching 60% confluence, cells were passaged in a 1:4 split ratio with TrypLE™ Express solution and passages between P12-P28 were used. Both cell lines showed characteristics of tumorigenicity and differ in their pheno- and genotype [Wang Y et al 2020, Deer EL et al 2010].

S4. Endothelial cells. HUVECs (Human umbilical vein endothelial cells) were obtained from pooled donors (from up to four different umbilical cords). These cells were cultured on 75-cm^2 flasks at 37ºC, 5% CO_2 in Endothelial growth medium II supplemented with 10 ml ready-to-use growth supplement.

After reaching 80-90% confluence, cells were passaged in a 1:3 split ratio with accutase-solution (0.15 ml/cm^2) and were used at passages between P2-P6. Detached cells were seeded on 0.1% fibronectin-coated wells and allowed to growth until they reached 100% confluence (mechanistic studies) or on 0.1% fibronectin-coated 24-well-transwell inserts of 24-well-plates (migration studies).

S5. Analysis of cell surface proteins (CAM) by flow cytometry analysis. 100 µL of cell suspension were used for staining with fluorochrome labelled monoclonal anti-human **REAfinity™ antibodies** in V1-channel (CD104 (ß4-integrin)-Vioblue, CD 49d (Viobright V423)), B1-channel (CD106 (VCAM-1)-FITC, CD49f (a6-integrin)-VioBright FITC), B2-channel (CD62E (E-selectin)-PE, VEGFR-PE), B4-channel (CD18 (ß2-integrin)-PE-Vio770), R1-channel (CD29 (ß1-integrin), CD54 (ICAM-1)-APC) and R2-channel (CD49c (α3-integrin)-APC-Vio770) and B3-channel for live-dead exclusion with propidium iodide solution (PI). Fluorochrome labelled IgG1-REAfinity™ antibodies were used as isotype controls. Compensation matrix for multicolour-measurement to avoid spillover was presented in Supplemental Table 3. Briefly, 100 µl cell suspension was stained for 10 min at 4^{0}C under dark conditions with anti-human **REAfinity™ or** IgG1-REAfinity™ **Isotype control or left unstained.** Reaction was stopped by adding 1000 µL cold MACS Running Buffer. Prior the flow cytometry measurements, 10 µl of PI was used for exclusion of dead cells. Thereafter, 100 µl cells suspension was measured automatically by flow cytometry with MACS Quant 10 (MQ10) and the quantification was performed using the MACSQuantify Version 2.13.2 software (Miltenyi, Bergisch-Gladbach, Germany) by comparing the mean fluorescence intensity (MFI). MFI was expressed as median with interquartile range (25th–75th). Representative Gating strategy and MFI quantification was given in Supplemental Figure 2.

S6. Cytokine determination. The secretion of tumor necrosis factor-α (TNF-α), interleukin-1ß (IL-1ß), IL-8 and VEGF present in the supernatant of pancreatic cancer cells and unstimulated HUVECs was measured colorimetrically using sandwich ELISAs with HRP-conjugate in the Digicsan Reader (Asys, Austria) according to the manufacturer's instructions. After the cultivation, the particulates were removed from the cell culture supernatant immediately by centrifugation (1,500xg, 5 min, RT) and samples were aliquoted and stored at -20°C with avoiding repeated freeze-thaw cycles. Quantification of secreted proteins from cells was performed using the Microwin software Version 2.15 and concentrations were given as pg/mL. The minimum detectable dose (MDD) of human TNF-α was 11.3 pg/mL, of IL-8 was 14.7 pg/mL, of human VEGF was 10.6 pg/mL, and of IL-1ß was 6.9 pg/mL. The optical density (OD) of each well was measured within 30 min after stopping the HRP-enzymatic reaction using the microplate reader at 450 nm. Concentrations were determined based on a calibration curve using standards for all cytokines. Protein secretion was expressed as are expressed as median with interquartile range (25th–75th).

S7. p65 and FAK activation measured by ELISA. Commercially available immunosorbent assays for FAK and NF-kB were used to determine phosphorylated levels of FAK at tyrosine residue 397(FAK [pY397]) in prepared cell lysates and the InstantOne™ ELISA for measurement of phosphorylated human NF-kB p65 in cell lysates. Cells were lysed using the recommended cell extraction buffer after incubation with plasma extracts and lysates were collected and stored at −80°C. The standard protocol of the manufacturer was followed thereafter. OD was measured at 450 nm and the expression of pFAK and p65 protein was calculated using a four-parameter algorithm providing the best standard curve fit. The background absorbance was subtracted from all data points, including standards, unknowns and controls prior to plotting. The results are expressed as mean ± SD.

S8. Determination of ROS with carboxy-H_2-DCFDA (5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein di-acetate). ROS determination and sample preparation have been described previously (5). Cellular ROS generation was determined by incubating cells in medium with 5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (carboxy-H_2-DCFDA). As noted by the manufacturer, an increase in carboxy-H_2-DCFDA fluorescence is primarily due to the reaction with intracellular ROS. The cell-permeable carboxy-H_2-DCFDA diffuses into cells and was retained in the intracellular level after cleavage by intracellular esterases. Upon oxidation by ROS, the non-fluorescent carboxy-H_2-DCFDA is converted to the highly fluorophore DCF (Ex 485 nm/Em 518 nm). Therefore, cells were grown in media with supplements and allowed to adhere for 24h on 24-well plates. After replacing the media, cells were exposed to medium containing extracted plasma samples (pH 7.4) and allowed to grow for another 5h. Cells were washed twice with HBSS and were incubated at 37^{O}C with 5 µmol/L carboxy-H_2-DCFDA for 30 min. Fluorescence increment was monitored over time using the Ascent microplate fluorescence reader. Fluorescence intensity (FI) after 30 min was subtracted from bassline values and the resulting δFI was expressed as δFI/1x10^5 cells. The results are expressed as mean ± SD.

S9. Targeted metabolomics UHPLC-MS/MS analysis. Mobile phases were based on positive and negative detection ion modes and were performed in separated runs in scheduled multiple reaction monitoring (sMRM). Positive ion mode consisted of water and acetonitrile and both of them contained 0.5% formic acid, while the negative ion mode consisted of water containing 0.1% formic acid and 10 mM ammonium formate and acetonitrile. This targeted metabolomics analysis was able to detect and quantify more than 1,000 metabolites in a single short run using volumes as low as 2 µl of the bio-fluid samples. Metabolites were identified by using commercial standards if available or by using their predicted nominal masses, optimizing their fragmentation conditions and estimating their quantity by using the calibration curves of other structurally similar metabolites and isomers. Analytical validation was optimized by assessing the linearity of the calibration curves at 12 different concentration levels between 0.1-10,000 µg/L. More information about validation and analytic performance of the method can be found in previous publications [Behrendt et al 2022]. Quality controls composed by a pool of

samples from the study were processed following the same procedures every 20 samples. Analyst 1.6.2 and Sciex OS software by Sciex were used for data acquisition and data processing, respectively. Finally, the coefficients of variation for areas, retention times and peak widths of the internal standards added to samples were calculated for analytical reproducibility assessment.

S10. Preprocessing of the metabolomics data. Metabolites with more than 30% of missing values were excluded from the analysis. For each metabolite, we calculated the fold change (FC) ratio between its concentration at the end of dietary intervention and its baseline measurement. Normal distribution of log_2FC in metabolites concentration was visually inspected. Afterwards, distances to the group centroid were computed based on Euclidean distances to remove outliers from the data matrix (±1.5×IQR). We used the R/Bioconductor package 'POMA' for preprocessing (10.1371/journal.pcbi.1009148).

S11. Basal expression of adhesion molecules on pancreatic cancer cells and secretion of cytokines. The expression of adhesion molecules such as integrins and selectins on cancer cells is one of the most important reasons of their migratory potential and therefore, their aggressivity. Circulating cancer cells were able to migrate across the endothelial barrier and settle down to establish a secondary tumour. α- and ß-integrins, CAMs such as ICAM and VCAM as well as E-SEL (E-selectins) are targets for therapeutics to reduce migration and inhibit metastatic processes. Here, we investigated whether extracted plasma metabolites have an influence on expression levels of pancreatic cancer cells because we have previously shown, that only PANC-1 migration, but not AsPC-1 migration was reduced by incubating cells with plasma metabolites isolated from blood of volunteers who received a short-term ACN ingestion [Kuntz S et al 2017]. Comparing surface markers on the pancreatic cancer cell lines PANC-1 and AsPC-1, significant differences were observed. Compared to AsPC-1, PANC-1 expressed significantly higher levels of α3-integrin (398 ± 14), α4-integrin (117 ± 8), ß1-integrin (1623 ± 73), ß2-integrin (35 ± 2), ESEL (53 ± 3), ICAM-1 (361 ± 26) and VEGR-R (18 ± 2). In case of α3-integrin, ß1-integrin, and ICAM-1, the expression levels were 12-fold, 5-fold and 90-fold higher on PANC-1 than on AsPC-1 cells (Supplementary Figure 3). Adhesion of cancer cells to endothelial cells were not only influenced by the expression levels of CAMs, their secretion of cytokines and VEGF could also influence the capability of migration. As shown in Supplementary Figure 4, secretion of TNF-α, IL-1ß and VEGF from PANC-1 was significantly higher than the secretion from AsPC-1. After 36-h cultivation, TNF-α concentration in the supernatant of cultivated PANC-1 was 128 ± 11 pg/ml in comparison to that of AsPC-1 with 25 ± 2.9 pg/ml. This was also seen for IL-1ß (22 ± 1.9 to 15 ± 0.8) and VEGF (35 ± 1.2 to 19 ± 4.3), whereas IL-8 secretion was not different between both pancreatic cancer cell lines (52 ± 1.2 to 53 ± 4.8).

S12. Basal expression of adhesion molecules on endothelial cells and CYTOmix-induced expression. In addition to the expression of CAMs, the so-called microclimate of the tumour plays an

essential role in the migration process of single tumour cells. In this context, the secretion of tumour cell associated cytokines which act on the endothelial barrier is another aspect that influences tumour aggressiveness. Binding of tumour cells with their CAMs to endothelial cells via the corresponding receptors could also be a result of the cancer cell specific microclimate. In order to investigate the effects of cytokines secreted from pancreatic cancer cells on HUVECs, HUVECs were exposed to a mixture of cancer cell specific cytokines (CytoMIX), which were detected before (see S11). Thereafter, expression levels of CAMs under basal und CytoMIX-stimulated cells were compared. As shown in Supplementary Figure 5, surface receptors on HUVECs under basal unstimulated conditions were expressed at very low levels. After a 36h stimulation with AsPC-1- CytoMIX (called `C1´) and PANC-1- CytoMIX (called ´C2´), only ß4-integrin and ICAM-1 expression were significantly enhanced compared to basal levels. AsPC-1- CytoMIX increased ß4-integrin and ICAM-1 0.2-fold and 2-fold, whereas the PANC-1- CytoMIX enhanced PANC-1 ß4-integrin and ICAM-1 0.3-fold and 9-fold. Concerning all other markers, AsPC-1- and PANC-1- CytoMIX have no significant effects on expression levels or other receptors on HUVECs (Supplementary Figure 5).

Supplementary Table 1: List of materials, biochemicals and kits used for cell culture assays

1290 Infinity UHPLC system	Agilent, XXX, U.S.A.
24-well plates	GreinerBioOne, Berlin, Germany
6-well plates	GreinerBioOne, Berlin, Germany
Accutase-solution	Promocell GmbH, Heidelberg, Germany
anti-human **REAfinity™ antibodies**	Miltenyi, Bergisch Gladbach, Germany
Ascent microplate fluorescence reader	ThermoScientific, Karlsruhe, Germany
AsPC-1	**ATCC (**LGC Standards GmbH), Wesel, Germany
Carboxy-H_2-DCFDA	Invitrogen GmbH, Darmstadt, Germany
CytoSelect™ 24-well Cell Migration Assay (8 µm), Colorimetric	CellBiolabs, San Diego, U.S.A.
Digicsan Reader	Asys, Eugendorf, Austria
DMEM (Dulbecco's modified Eagle's medium)	Invitrogen GmbH, Darmstadt, Germany
Endothelial growth medium II	Promocell GmbH, Heidelberg, Germany
FAK (Phospho) [pY397] Human ELISA Kit	ThermoScientific, Karlsruhe, Germany
Fetal bovine serum	PAA Laboratories GmbH, Cölbe, Germany
Fibronectin solution	Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany
Formic acid	LGC Promochem, Wesel, Germany
GraphPad Prism Version 9	GraphPad Software, LLC, San Diego, U.S.A.
H1 microplate fluorescence reader	Biotec GmhH, Karlsruhe, Germany
HBSS	Invitrogen GmbH, Darmstadt, Germany
HUVECs	Promocell GmbH, Heidelberg, Germany
IgG1-REAfinity™ antibodies	Miltenyi, Bergisch Gladbach, Germany
Interleukin (IL-8) ELISA	ThermoScientific, Karlsruhe, Germany
Interleukin-1ß (IL-1ß) ELISA	ThermoScientific, Karlsruhe, Germany
Ion Drive Turbo V ion source	Sciex, XXX, U.S.A.
L-glutamine	Invitrogen GmbH, Darmstadt, Germany
Luna Omega Polar C18 column	Phenomenex, XXX, U.S.A.
MACS Running Buffer	Miltenyi, Bergisch Gladbach, Germany
MACS Quant 10 (MQ10)	Miltenyi, Bergisch Gladbach, Germany
Methanol	ThermoScientific, Karlsruhe, Germany
Micro-Spin Eppendorf filter (pore size 0.2 µm)	Eppendorf, Hamburg, Germany
Millicell® ERS volt-ohmmeter	Millipore Corporation, Bedford, U.S.A.
NF-kB InstantOne™ ELISA	ThermoScientific, Karlsruhe, Germany
SPE cartridge Oasis-HLB (5 mL/200 mg)	Waters, Inc., Eschborn, Germany
PANC-1	**ATCC (**LGC Standards GmbH), Wesel, Germany
PBS	Invitrogen GmbH, Darmstadt, Germany

Propidium iodide solution (PI)	Miltenyi, Bergisch Gladbach, Germany
R statistical software	R foundation, Vienna, Austria
RPMI 1640 medium (Roswell Park Memorial Institute)	Invitrogen GmbH, Darmstadt, Germany
Sodium pyruvate	Invitrogen GmbH, Darmstadt, Germany
TrypLE™ Express solution	ThermoScientific, Karlsruhe, Germany
Tumor necrosis factor-α (TNF-α) ELISA	ThermoScientific, Karlsruhe, Germany
Vascular endothelial growth factor (VEGF) ELISA	R&D Systems, Heidelberg, Germany

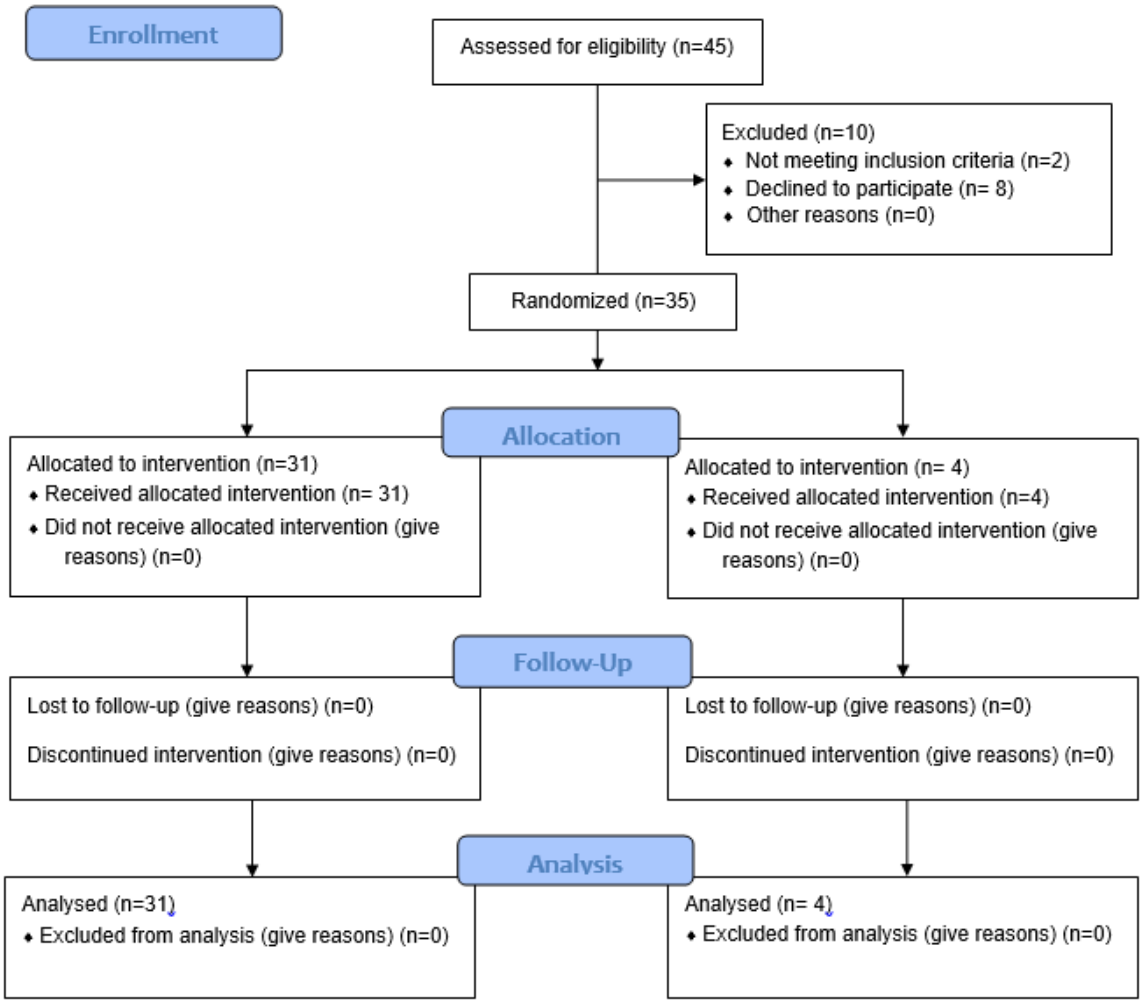
Supplementary Table 2: CONSORT 2010 checklist of information to include when reporting a randomised trial

Section/Topic	Item No	Checklist item	Reported on page No
Title and abstract			
	1a	Identification as a randomised trial in the title	1
	1b	Structured summary of trial design, methods, results, and conclusions (for specific guidance see CONSORT for abstracts)	2
Introduction			
Background and objectives	2a	Scientific background and explanation of rationale	2-3
	2b	Specific objectives or hypotheses	3
Methods			
Trial design	3a	Description of trial design (such as parallel, factorial) including allocation ratio	3, previously published [Behrendt et al 2022]
	3b	Important changes to methods after trial commencement (such as eligibility criteria), with reasons	No changes
Participants	4a	Eligibility criteria for participants	3, previously published [Behrendt et al 2022]
	4b	Settings and locations where the data were collected	3, previously published [Behrendt et al 2022]
Interventions	5	The interventions for each group with sufficient details to allow replication, including how and when they were actually administered	3, previously published [Behrendt et al 2022]
Outcomes	6a	Completely defined pre-specified primary and secondary outcome measures, including how and when they were assessed	4-5
	6b	Any changes to trial outcomes after the trial commenced, with reasons	No
Sample size	7a	How sample size was determined	3, previously published [Behrendt et al 2022]
	7b	When applicable, explanation of any interim analyses and stopping guidelines	
Randomisation:			
Sequence generation	8a	Method used to generate the random allocation sequence	3, previously published [Behrendt et al 2022]
	8b	Type of randomisation; details of any restriction (such as blocking and block size)	No block size
Allocation concealment mechanism	9	Mechanism used to implement the random allocation sequence (such as sequentially numbered containers), describing any steps taken to conceal the sequence until interventions were assigned	3, previously published [Behrendt et al 2022]
Implementation	10	Who generated the random allocation sequence, who enrolled participants, and who assigned participants to interventions	3, previously published [Behrendt et al 2022]
Blinding	11a	If done, who was blinded after assignment to interventions (for example, participants, care providers, those assessing outcomes) and how	3, previously published [Behrendt et al 2022]
	11b	If relevant, description of the similarity of interventions	no similarity
Statistical methods	12a	Statistical methods used to compare groups for primary and secondary outcomes	5
	12b	Methods for additional analyses, such as subgroup analyses and adjusted analyses	5
Results			

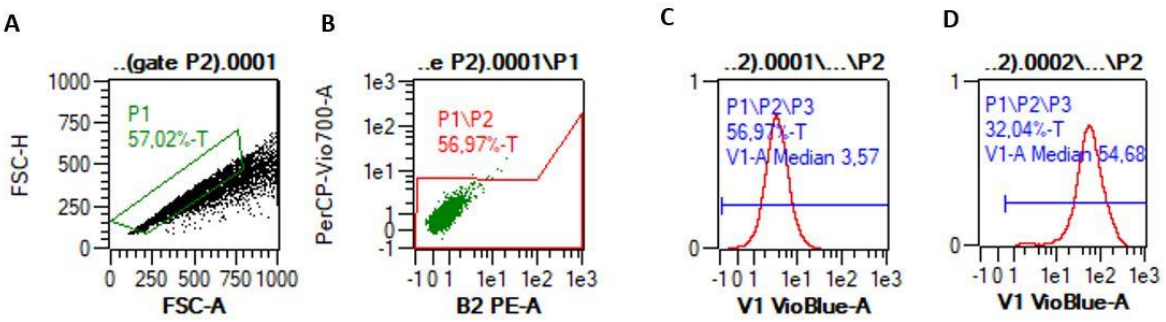
Participant flow (a diagram is strongly recommended)	13a	For each group, the numbers of participants who were randomly assigned, received intended treatment, and were analysed for the primary outcome	Supplemental Data
	13b	For each group, losses and exclusions after randomisation, together with reasons	Supplemental Data
Recruitment	14a	Dates defining the periods of recruitment and follow-up	3, previously published [Behrendt et al 2022]
	14b	Why the trial ended or was stopped	
Baseline data	15	A table showing baseline demographic and clinical characteristics for each group	6, previously published [Behrendt et al 2022]
Numbers analysed	16	For each group, number of participants (denominator) included in each analysis and whether the analysis was by original assigned groups	6-7, Supplements
Outcomes and estimation	17a	For each primary and secondary outcome, results for each group, and the estimated effect size and its precision (such as 95% confidence interval)	6-7, Supplements
	17b	For binary outcomes, presentation of both absolute and relative effect sizes is recommended	
Ancillary analyses	18	Results of any other analyses performed, including subgroup analyses and adjusted analyses, distinguishing pre-specified from exploratory	6-7, Supplements
Harms	19	All important harms or unintended effects in each group (for specific guidance see CONSORT for harms)	
Discussion			
Limitations	20	Trial limitations, addressing sources of potential bias, imprecision, and, if relevant, multiplicity of analyses	7
Generalisability	21	Generalisability (external validity, applicability) of the trial findings	7
Interpretation	22	Interpretation consistent with results, balancing benefits and harms, and considering other relevant evidence	7-8
Other information			
Registration	23	Registration number and name of trial registry	3
Protocol	24	Where the full trial protocol can be accessed, if available	3, previously published [Behrendt et al 2022]
Funding	25	Sources of funding and other support (such as supply of drugs), role of funders	1,8

Supplementary Table 3: MultiColor 8 x 8 compensation matrix. Anti-REA MACS Comp Bead Kit was used for compensation of fluorescence spillover of fluorochrome-conjugated REAfinity™ antibodies. Diluted (1:10 or 1:50) REAfinity™ fluorochrome-conjugated antibodies were incubated for 10 min in dark at room temperature with 50 µl anti-REA `Comp Beads´ or 50 µl blank `Comp Beads´. After incubation, 1 ml MACSQuant running buffer was added to the reaction and fluorescence spillover was automated measured with MQ10. Spillover matrix was saved as instrument settings for PANC-1, AsPC-1 and HUVEC. Individual fluorescence signals were organized in columns and fluorescence channels in rows (8x8).

	VioBlue	VioGreen	FITC	PE	PerCP-Vio770	PE-Vio770	APC	APC-Vio770
V1	1.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
V2	0.0	1.0	0.0	0.0	0.0	0.002	0.0	0.0
B1	0.0	0.0	1.0	0.029	0.0	0.007	0.0	0.0
B2	0.0	0.0	0.08	1.0	0.0	0.028	0.0	0.0
B3	0.0	0.0	0.016	0.207	1.0	0.0	0.013	0.001
B4	0.0	0.0	0.002	0.022	0.0	1.0	0.002	0.014
R1	0.0	0.0	0.0	0.0	0.0	0.003	1.0	0.053
R2	0.0	0.0	0.0	0.0	0.0	0.114	0.144	1.0



Supplementary Figure 1: CONSORT flowchart diagram.



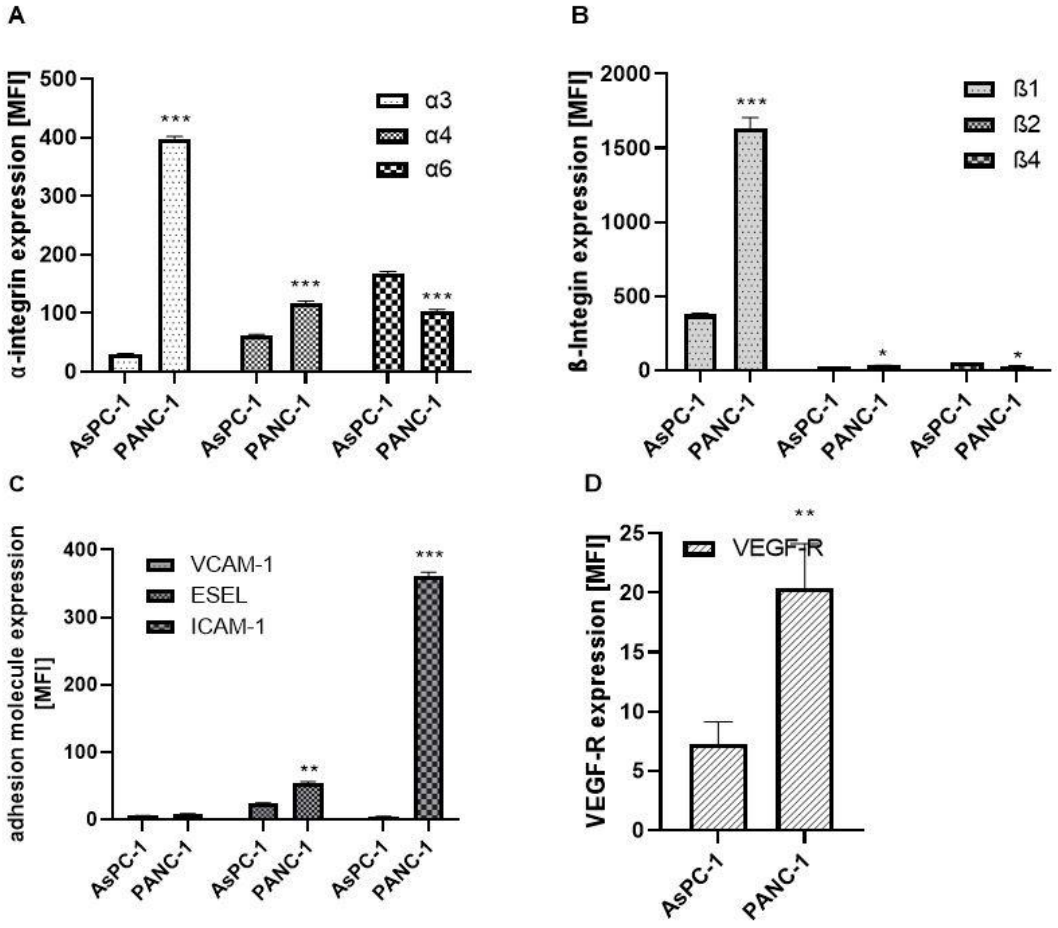
E

Path	V1 VioBlue-A Mean	B1 FITC-A Mean	B2 PE-A Mean	PE-Vio770-A Mean	R1 APC-A Mean	APC-Vio770-A Mean
P1\P2\P3	4,09	2,61	0,64	0,34	0,50	0,53
P1\P2\P4	4,09	2,61	0,64	0,34	0,50	0,53
P1\P2\P5	4,09	2,61	0,64	0,34	0,50	0,53
P1\P2\P6	4,09	2,61	0,64	0,34	0,50	0,53
P1\P2\P7	4,09	2,61	0,64	0,34	0,50	0,53
P1\P2\P8	4,09	2,61	0,64	0,34	0,50	0,53

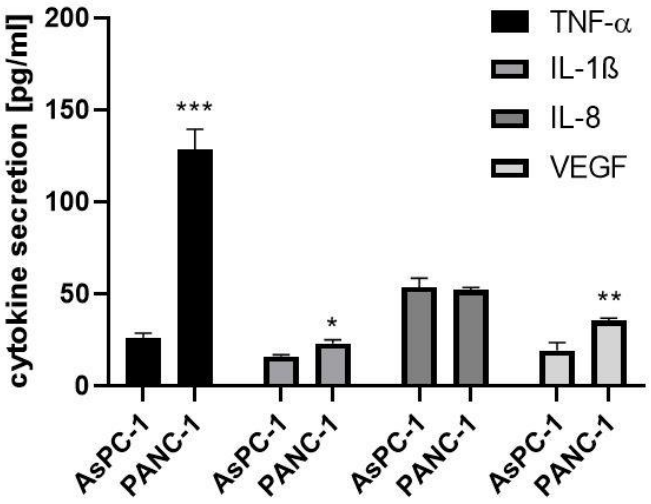
F

Path	V1 VioBlue-A Mean	B1 FITC-A Mean	B2 PE-A Mean	PE-Vio770-A Mean	R1 APC-A Mean	APC-Vio770-A Mean
P1\P2\P3	63,21	12,93	30,70	31,44	10,15	37,89
P1\P2\P4	63,24	13,13	31,26	31,95	10,28	38,16
P1\P2\P5	64,21	14,57	35,89	36,04	11,43	39,82
P1\P2\P6	64,05	14,45	34,22	36,17	11,36	39,66
P1\P2\P7	64,29	14,70	34,90	36,50	11,75	40,03
P1\P2\P8	63,26	12,97	30,82	31,57	10,17	37,97

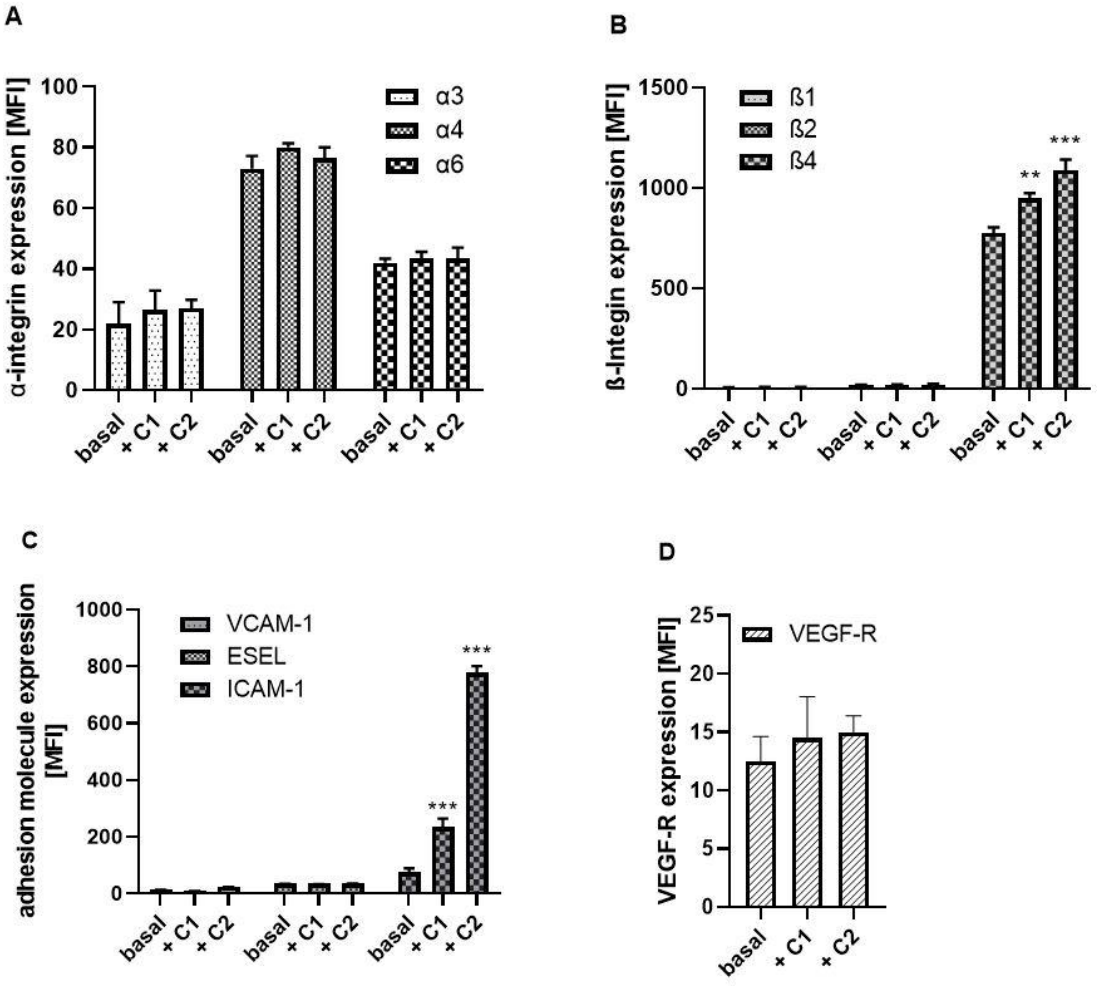
Supplementary Figure 2. Gating strategy and quantification. (A) Cells were gated by FSC(A) *vs.* FSC(H) plot: Gating the cells that have an equal area and height, thus removing clumps (greater FSC(A) relative to FSC(H)) and debris (very low FSC) [P1-marker]; **(B)** PerCP-Vio770 *vs.* B2-channal plot: broad selection of living cells (P2 marker) and exclusion of dead cells stained with propidium iodide [P2-marker]; **(C)** Histogram plots of unstained cells in V1-channel (CD104); **(D)** Histogram plots of unstained cells in V1-channel (CD104); Histogram markers were used to quantify the main fluorescence intensity (means with SD or median with SD) for **E)** unstained and **(F)** stained cells [representative staining panel 1].



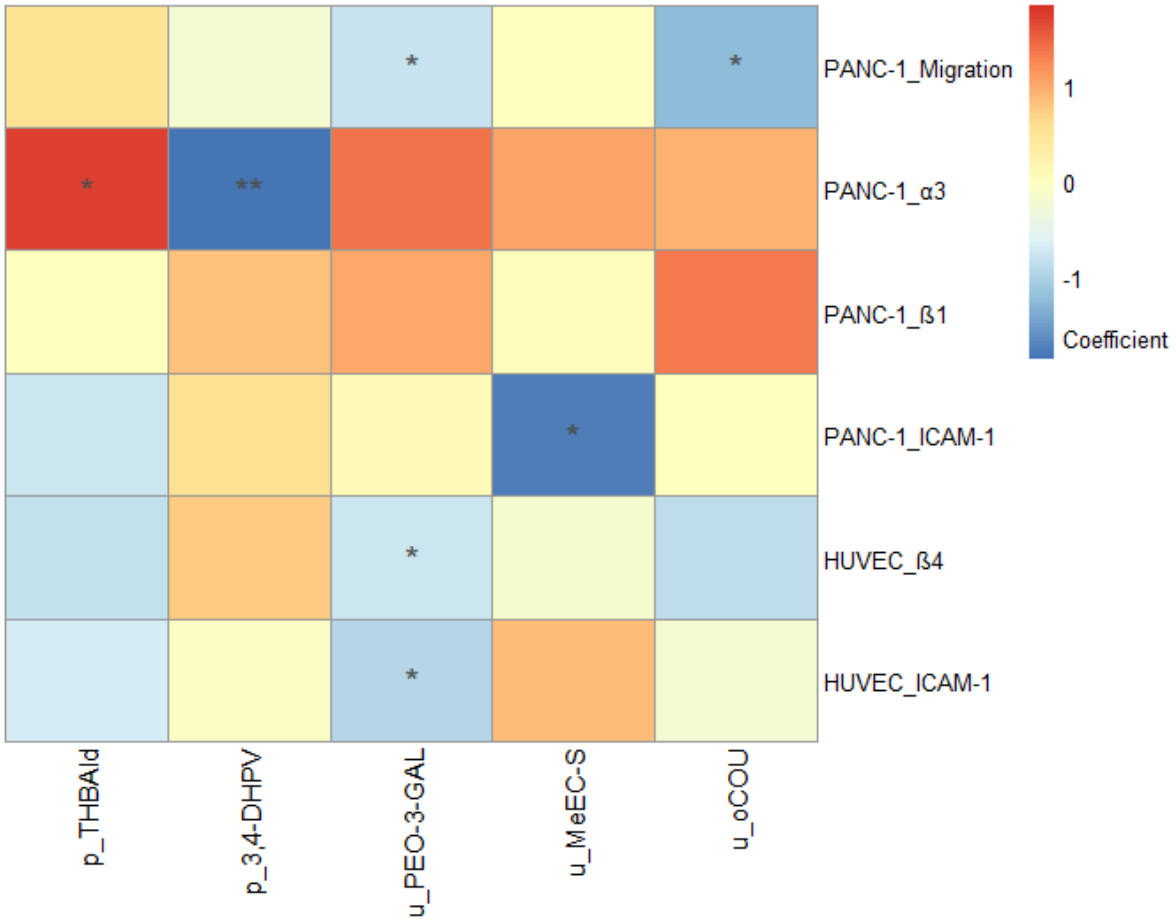
Supplementary Figure 3. Protein expression of adhesion molecules and markers on PANC-1 and AsPC-1 *in vitro*. Expression levels of α-integrins **(A)**, ß-integrins **(B)**, CAMs and E-SEL **(C)** and VEGF-R **(D)** on the cell surface of pancreatic cancer cells were measured under basal conditions. PANC-1 and AsPC-1 cells were seeded onto 6-well plates ($1x10^5$ cells/mL) and allowed to adhere for 24 h. Thereafter, medium was changed and cells grow for further 36h in corresponding medium. After incubation, medium was removed, cells were washed twice with PBS, detached with 1 ml TrypLE™ Express solution and centrifuged at 300xg for 5 min at RT. Cells were resuspended in MACS-Running buffer and expression levels were measured after cells were stained with anti-human REA-antibodies coupled with fluorochromes by flow cytometry as described in the Methods section (see 2.4.2.). Values are expressed as means of main fluorescence intensity (MFI) with standard deviation (means ± SD). Significant differences between the pancreatic cancer cell lines were calculated with One-way ANOVA and values were different with $P^{*} < 0.05$, $P^{**} < 0.01$ and $P^{***} < 0.001$ (n=3 in duplicates).



Supplementary Figure 4. Secretion of cytokines and VEGF from PANC-1 and AsPC-1 *in vitro*. PANC-1 and AsPC-1 were seeded onto 6-well plates (1 x 10^5 cells/mL) and allowed to adhere for 24h. Thereafter, medium was changed and cells grow for further 36h in corresponding medium. After incubation, supernatant was removed and centrifugated (1,500xg, 5 min, RT). Protein levels were measured by ELISA as described in the Methods section (see 2.4.2.). Values are expressed as means of secreted proteins as pg/ml ± standard deviation (means ± SD). Significant differences between the pancreatic cancer cell lines were calculated with One-way ANOVA and values were different with *P**< 0.05, *P***< 0.01 and *P****< 0.001 to corresponding controls (n=3 in duplicates).



Supplementary Figure 5. Expression of adhesion molecules and markers on basal (unstimulated) and CYTOmix-stimulated HUVEC *in vitro*. Expression of α-integrins **(A)**, ß-integrins **(B)**, CAMs and ESEL **(C)** and VEGF-R **(D)** on cell surface of HUVECs were measured under basal (unstimulated) or stimulated conditions (´C1´ or ´C2´). HUVECs were seeded onto fibronectin (0.1%)-coated 6-well plates (5 x 10^5 cells/mL) and allow to grow to confluence. After reaching confluence, medium was changed and cells grow for further 36h in corresponding medium with AsPC-1-CYTOmix (C1: 25 pg/ml TNF-α, 15 pg/ml IL-1ß, 50 pg/ml IL-8, 20 pg/ml VEGF) or PANC-1-CYTOmix (C2: 130 pg/ml TNF-α, 25 pg/ml IL-1ß, 50 pg/ml IL-8, 35 pg/ml VEGF). Thereafter, cells were washed twice with PBS, detached with 1 ml accutase-solution and centrifuged at 220xg for 3 min at RT. Cells were resuspended in MACS-Running buffer and expression levels were measured after cells were stained with anti-human REA antibodies coupled with fluorochromes by flow cytometry as described in the Methods section (see 2.4.2.). Values are expressed as means of MFI ± standard deviation (means ± SD). Significant differences between basal and CYTOmix-stimulated cells were calculated with One-way ANOVA and values were different with *P**< 0.05, *P***< 0.01 and *P****< 0.001 (n=3 in duplicates).



Supplementary Figure 6. Heatmap showing the association between cancer cells analyses and plasma and urine metabolites altered by the ACN-rich juice. Assessed using linear mixed models with participants as random effects adjusting by age, sex, and treatment. p_: plasma metabolite; u_: urine metabolite; THBAld: 2,4,6-trihydroxybenzaldehyde; 3,4-DHPV: 3',4'-dihydroxyphenyl-γ-valerolactone; PEO-3-GAL: peonidin 3-galactoside; MeEC-3-S: 3'-methyl(epi)catechin sulphate; oCOU: o-coumaric acid. *: *p*-value <0.05; **: P-value <0.01. Metabolites included in the heatmap are those with at least one statistically significant association.

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5.2.3. Associations between long-term intake of an ACN-rich juice and fecal metabolomics in the ATTACH study

Seven metabolites in feces from the 330 metabolites that were evaluated were significantly associated with ACN-rich juice intake as shown in Figure 5.1. Among these metabolites, glutaric acid, vanillic acid (VA), homovanillyl alcohol (HVAlc) and four parents ACN: cyanidin-3-arabinoside (cya-3-ara), peonidin-3-glucoside (peo-3-glc), malvidin-3-glucoside (mal-3-glc) and petunidin 3-glucoside (pet-3-glc) were significantly altered in feces.

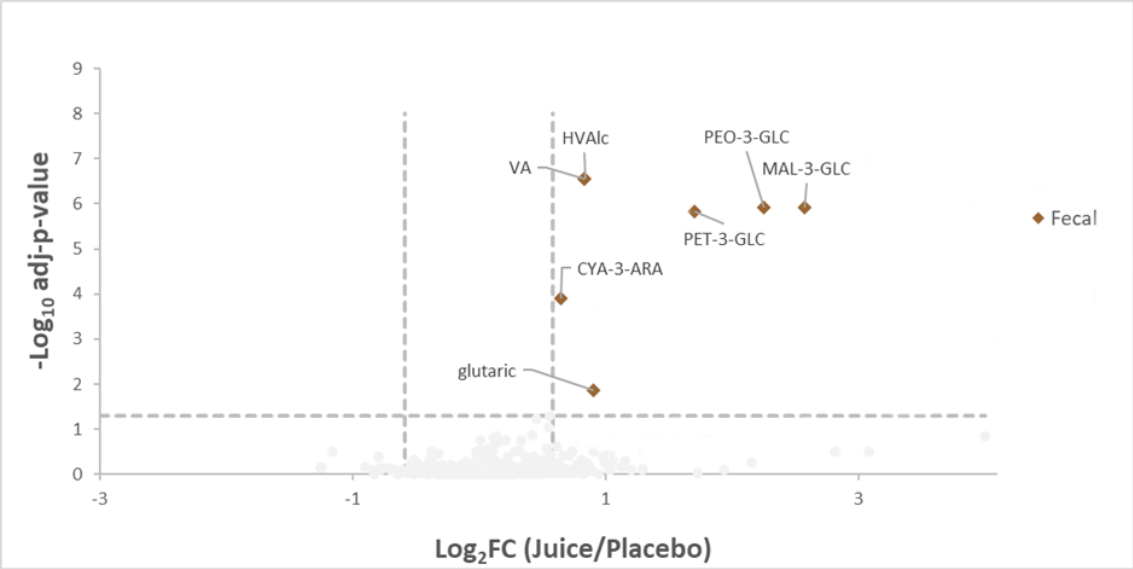

Figure 5.1: Volcano plot of log_2FC vs log_{10} FDR-adjusted *p-value*, showing the metabolites that are significantly associated with ACN intake in feces. The FC value of each metabolite corresponds to the difference between log_2FCpost/pre during ACN-rich juice and log_2FCpost/pre during placebo intervention. To determine the effect of the ACN-rich juice, LMM was used with treatment (juice/placebo), sex and age as fixed factors and subject ID as random factor. *P-values* were calculated and adjusted using FDR. VA: vanillic acid; HVAlc: homovanillyl alcohol; PEO-3-GLC: peonidin-3-glucoside; CYA-3-ARA: cyanidin-3-arabinoside; MAL-3-GLC: Malvidin-3-glucoside; PET-3-GLC: Petunidin-3-glucoside.

5.3. Observation study: DCH-NG MAX study

5.3.1. Publication 4. Dietary sources of anthocyanins and their association with metabolome biomarkers and cardiometabolic risk factors in an observational study.

Hamza Mostafa, Tomás Meroño, Antonio Miñarro, Alex Sanchez-Pla, Fabián Lanuza, Raul Zamora-Ros, Agnetha Linn Rostgaard-Hansen, Núria Estanyol-Torres, Marta Cubedo-Cullere, Anne Tjønneland, Rikard Landberg, Jytte Halkjær, Cristina Andres-Lacueva.
Nutrients 2023, 15, 1208. https://doi.org/10.3390/nu15051208

Journal impact factor: 6.706
Journal quartile: Q1 (Category: Nutrition & Dietetics, 15/90)

Abstract:

Anthocyanins (ACNs) are (poly)phenols associated with reduced cardiometabolic risk. Associations between dietary intake, microbial metabolism, and cardiometabolic health benefits of ACNs have not been fully characterized. Our aims were to study the association between ACN intake, considering its dietary sources, and plasma metabolites, and to relate them with cardiometabolic risk factors in an observational study. 1,351 samples from 624 participants (55% female, mean age: 45±12 years-old) enrolled in the MAX-study were studied using a semi-targeted metabolomic analysis. 24-hour dietary recalls were used to collect dietary data at baseline, 6 and 12 months. ACN content of foods was calculated using Phenol Explorer and foods were categorized into 8 food groups (berries, wine, non-alcoholic drinks, other fruits, vegetables, bakery, dairy products with berries, and mixed dishes). Median intake of total ACNs was 1.6mg/day (mean: 26.3 mg/day). Using mixed graphical models, ACNs from different foods showed specific associations with plasma metabolome biomarkers. Combining these results with a regression analysis, metabolites associated with ACNs intake were: salsolinol-sulfate, 4-methylcatechol-sulfate, linoleoyl-carnitine and two valerolactones. Salsolinol-sulfate and 4-methylcatechol-sulfate, both related to intake of ACNs mainly from berries, were inversely associated with visceral adipose tissue volume. In conclusion, plasma metabolome biomarkers of dietary ACNs depended on the dietary source and some of them, such as salsolinol-sulfate and 4-methylcatechol-sulfate may provide a mechanistic link between berry intake and its cardiometabolic health benefits.

Resumen:

Las antocianinas (ACNs) son (poli)fenoles cuyo consumo se ha asociados con una reducción del riesgo cardiometabólico. Las asociaciones entre la ingesta de ACNs, su metabolismo microbiano y los beneficios para la salud cardiometabólica no se han caracterizado completamente. Nuestros objetivos fueron estudiar la asociación entre la ingesta de ACN, considerando sus fuentes dietéticas, y los metabolitos plasmáticos, y relacionarlos con los factores de riesgo cardiometabólico en un estudio observacional. Se estudiaron 1.351 muestras de 624 participantes (55% mujeres, edad media: 45 ± 12 años) inscritos en el estudio MAX utilizando un análisis metabolómico semidirigido. Se utilizaron recordatorios de 24 horas para recopilar datos dietéticos al inicio, a los 6 y 12 meses. El contenido de ACN de los alimentos se calculó utilizando Phenol Explorer y se categorizaron en 8 grupos de alimentos (bayas, vino, bebidas sin alcohol, otras frutas, verduras, panadería, productos lácteos con bayas y platos mixtos). La mediana de ingesta de ACN totales fue de 1,6 mg/día (media 26.3 mg/día). Utilizando modelos gráficos mixtos, las ACN de diferentes alimentos mostraron asociaciones específicas con biomarcadores del metaboloma plasmático. Combinando estos resultados con un análisis de regresión censurada, los metabolitos asociados con la ingesta de ACN fueron: sulfato de salsolinol, sulfato de 4-metilcatecol, linoleoil-carnitina, y dos valerolactonas. El sulfato de salsolinol y el sulfato de 4-metilcatecol, ambos relacionados con la ingesta de ACN principalmente de bayas, se asociaron inversamente con el volumen del tejido adiposo visceral. En conclusión, los biomarcadores del metaboloma plasmático de las ACN dietéticos dependieron de la fuente dietética y algunos de ellos, como el sulfato de salsolinol y el sulfato de 4-metilcatecol, pueden proporcionar un vínculo mecanístico entre la ingesta de bayas y la mejora de la salud cardiometabólica.

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Dietary Sources of Anthocyanins and Their Association with Metabolome Biomarkers and Cardiometabolic Risk Factors in an Observational Study

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Citation: Mostafa, H.; Meroño, T.; Miñarro, A.; Sánchez-Pla, A.; Lanuza, F.; Zamora-Ros, R.; Rostgaard-Hansen, A.L.; Estanyol-Torres, N.; Cubedo-Culleré, M.; Tjønneland, A.; et al. Dietary Sources of Anthocyanins and Their Association with Metabolome Biomarkers and Cardiometabolic Risk Factors in an Observational Study. *Nutrients* **2023**, *15*, 1208. https://doi.org/10.3390/nu15051208

Academic Editors: Sonia de Pascual-Teresa and Luis Goya

Received: 9 February 2023
Revised: 24 February 2023
Accepted: 25 February 2023
Published: 28 February 2023

Abstract: Anthocyanins (ACNs) are (poly)phenols associated with reduced cardiometabolic risk. Associations between dietary intake, microbial metabolism, and cardiometabolic health benefits of ACNs have not been fully characterized. Our aims were to study the association between ACN intake, considering its dietary sources, and plasma metabolites, and to relate them with cardiometabolic risk factors in an observational study. A total of 1351 samples from 624 participants (55% female, mean age: 45 ± 12 years old) enrolled in the DCH-NG MAX study were studied using a targeted metabolomic analysis. Twenty-four-hour dietary recalls were used to collect dietary data at baseline, six, and twelve months. ACN content of foods was calculated using Phenol Explorer and foods were categorized into food groups. The median intake of total ACNs was 1.6mg/day. Using mixed graphical models, ACNs from different foods showed specific associations with plasma metabolome biomarkers. Combining these results with censored regression analysis, metabolites associated with ACNs intake were: salsolinol sulfate, 4-methylcatechol sulfate, linoleoyl carnitine, 3,4-dihydroxyphenylacetic acid, and one valerolactone. Salsolinol sulfate and 4-methylcatechol sulfate, both related to the intake of ACNs mainly from berries, were inversely associated with visceral adipose tissue. In conclusion, plasma metabolome biomarkers of dietary ACNs depended on the dietary source and some of them, such as salsolinol sulfate and 4-methylcatechol sulfate may link berry intake with cardiometabolic health benefits.

Keywords: metabolomics; anthocyanins; food matrix; diet; gut microbiota; berries; cardiometabolic health

1. Introduction

Anthocyanins (ACNs) are phytochemical compounds of the subclass of flavonoids in the broader (poly)phenol class, highly present in plant foods, such as berries, grapes, eggplants, and many other colored fruits and vegetables [1,2]. Most of the dietary ACNs reach the large intestine unaffected where they may affect both gut microbial composition

and microbial metabolism of ACNs [2,3]. Dietary ACNs and their microbial metabolites are suggested to play roles in the prevention and treatment of cardiometabolic diseases [4,5]. Microbial metabolites of ACNs have been shown to reach higher concentrations in the systemic circulation and may be more bioactive than the consumed ACNs per se [6]. Nonetheless, studies evaluating the association between ACN dietary intake and plasma concentrations of ACN-derived microbial metabolites in observational studies are lacking.

Sources of variability in ACN metabolism by the host and gut microbiota could be related to differences in consumed types and quantity of ACNs, as well as to a food matrix effect. Ultimately, these differences might be translated into different health effects of ACNs in response to their intake. Up to the moment, besides parent ACNs, such as cyanidin, delphinidin, malvidin, and petunidin, various metabolites derived from microbial and host metabolism (i.e., protocatechuic acid, syringic acid, and 4-hydroxybenzoic acid) have been associated with the consumption of berries in dietary intervention studies [7–9]. However, to the best of our knowledge, associations between the intake of ACNs, annotated according to their dietary sources, microbial metabolites, and cardiometabolic risk factors have not been studied in an observational study. Our hypothesis is that specific sets of microbial metabolites would be associated with dietary ACNs from different food sources and that these will display differential associations with cardiometabolic risk factors. The aims of this study were to evaluate the association between the intake of ACNs, considering their different dietary sources, and plasma metabolites. The aim was further to explore the associations between ACN-related metabolites and cardiometabolic risk factors in a subsample of the Danish Diet, Cancer, and Health-Next Generations (DCH-NG) MAX study [10].

2. Materials and Methods

2.1. Study Design and Subjects

We studied a validation subsample within the Diet, Cancer, and Health-Next Generations (DCH-NG) cohort: the DCH-NG MAX study. The DCH-NG was an extension of the previous cohort the Diet, Cancer, and Health (DCH) [10]. A sample of 39,554 participants was included in the DCH-NG involving biological children, their spouses, and grandchildren of the DCH cohort [11]. The DCH-NG MAX study recruited 720 volunteers with residency in Copenhagen, aged 18 years old or more, between August 2017 and January 2019. The major aims of the MAX study were to validate a semi-quantitative food frequency questionnaire against the twenty-four-hour dietary recalls (24-HDR) and to examine the plasma and urine metabolome reproducibility as well as gut microbial stability on a long-term scale. Biological samples, health examinations such as anthropometric and blood pressure measurements, and two questionnaires about lifestyle and dietary habits were collected at baseline, 6, and 12 months.

The DCH-NG cohort study was approved by the Danish Data Protection Agency (journal number 2013-41-2043/2014-231-0094) and by the Committee on Health Research Ethics for the Capital Region of Denmark (journal number H-15001257). The volunteers provided their written informed consent to participate in the study. All the details about clinical measurements, dietary and metabolomics data were previously detailed [11].

2.2. Anthropometric Measurements

Participants were asked to wear underwear and be barefoot for measuring height and weight using a wireless stadiometer and a body composition analyzer, respectively (SECA mBCA515, Hamburg, Germany). Height and weight were measured to the nearest 0.1 cm and 0.01 kg, respectively, and body mass index (BMI) was calculated. The waist circumference was measured twice at the midpoint between the lower rib margin and the iliac crest. A third measurement for the waist circumference was measured if the difference between the first two was more than 1 cm. Blood pressure and pulse rate were measured 3 times using the left arm, considering the measurement with the lower systolic blood pressure and its corresponding diastolic blood pressure value as valid. DEXA-validated bioimpedance instrument (SECA mBCA515, Germany) was used to estimate visceral adipose tissue volume.

2.3. Dietary Data

The 24-h dietary recalls (24-HDR) were recorded at baseline, 6 and 12 months using a Danish version of the web-based tool myfood24 (www.myfood24.org/) (7 February 2023) from Leeds University [12], containing almost 1600 Danish food items. All foods consumed the day before the examinations were reported by the participants in either grams or in standard portion size. The percentage of calories using the energy equivalents for carbohydrates, proteins, and fat was used to indicate the intake of macronutrients. Complex food products were appointed as recipes or dishes. The McCance and Widdowson's Food Composition Table [13], or recipes from the food frequency questionnaires in the DCH were used to have the standardized recipes [14].

Dietary Intake of Anthocyanins

Estimation of the intake of polyphenols from 24-HDRs was completed by a protocol using "in-house" software developed by the University of Barcelona, the Bellvitge Biomedical Research Institute (IDIBELL), and the Centro de Investigación Biomédica en Red (CIBER) ©. A link between all 24-HDR food items or ingredients and the foods from the Phenol-Explorer database was created [15]. The intake of individual (poly)phenols in mg/day was obtained and ACN consumption from separate foods were estimated as the sum of 71 individual ACNs included in Phenol-Explorer database. The estimated intake of dietary (poly)phenols of the DCH-NG MAX study has previously been described [16]. A total of 147 food items that contain ACNs were used to estimate the total dietary ACN intake as shown in the Supplementary Table S1. Intake of berries was estimated as the sum of foods with at least 50% of its composition or recipe made by berries. These include raw and frozen berries, berries marmalades or jams, and stewed berries. Dietary ACN intake related with the other foods were classified and added up according to the following food groups: dairy products with berries (including ice cream and yogurt), other fruits (i.e., plums, cherries, apples, etc.), non-alcoholic drinks (including fruit smoothies and juices), wines, vegetables, mixed dishes (meat or fish dishes with vegetables containing ACNs), and bakery (including pastry, biscuits, desserts, and waffles with berries or other ACN-containing preparations). (Supplementary Table S2). Intakes of foods not containing ACN were disregarded.

2.4. Blood Sampling, Analysis of Cardiometabolic Risk Factors and Metabolomics

Participants were instructed to maintain a fasting time of 1–9 h (mean fasting time: 5 h) during all the examination days. Blood samples were taken into Vacutainer tubes containing lithium heparin at baseline time 0 (n = 624), 6 months (n = 380), and 12 months (n = 349). Within 2 h of blood draw, plasma was obtained by centrifugation, and samples were stored at −80 °C. After that, plasma samples were delivered to the Danish National Biobank (DNB), where plasma was divided into aliquots and sent to University of Barcelona and kept at −80 °C until metabolomic analysis. Other blood measurements such as hemoglobin, A1c (HbA1c), serum lipids, and high sensitivity C reactive protein (hsCRP) were measured as described before [17].

2.4.1. Metabolomics Analysis of Plasma Samples

Repeated measures of the plasma metabolome at all three time points were used for metabolomics analysis. All the samples were prepared and analyzed using the targeted UPLC-MS/MS method described previously, with slight modifications [18,19]. Briefly, 100 µL of plasma was added into protein precipitation plates together with 500 µL cold acetonitrile containing 1.5 M formic acid and 10 mM of ammonium formate and were kept at −20 °C for 10 min to enhance protein precipitation. Then, positive pressure was applied to recover the extracts, which were taken to dryness and reconstituted with 100 µL of an 80:20 *v/v* water:aceto nitrile solution containing 0.1% *v/v* formic acid and 100 ppb of a mixture of 13 internal standards. Samples were then transferred to 96-well plates and analyzed by a targeted metabolomic analysis using an Agilent 1290 Infinity UPLC

system coupled to a Sciex QTRAP 6500 mass spectrometer, using the operating conditions described elsewhere [18]. The Sciex OS 2.1.6 software (Sciex, Framingham, MA, USA) was used for data processing.

2.4.2. Metabolomics Data Pre-Processing

The POMA R/Bioconductor package (https://github.com/nutrimetabolomics/POMA) (7 February 2023) was used for the pre-processing of metabolomics data [20]. Metabolites with more than 40% missing values, and those with a coefficient of variation (CV) > 30% in internal quality control were removed. K-nearest neighbor (KNN) algorithm and correction of batch effects using the ComBat function ("sva" R package) were used to impute the remaining missing values [21], while auto-scaling and Euclidean distances ($\pm 1.5\times$ Interquartile range) were used to normalize the data and remove the outliers, respectively. The final metabolomics dataset included the concentration of 408 plasma metabolites.

2.5. Statistical Analyses

For descriptive statistics, intake of total ACNs (irrespective of their dietary source) was categorized by tertiles using 0.3–8.9 mg as thresholds. Continuous variables following a normal distribution are shown as mean ± SD, and those following a skewed distribution are shown as median (p25–p75). Sociodemographic and clinical characteristics were compared across tertiles of ACNs intake using linear mixed models in a random intercepts model adjusted for age and sex. Associations between intake of ACN dietary sources and their association with cardiometabolic risk factors were tested using linear mixed models in random intercepts models adjusted for age, sex, and BMI.

First, associations between intake of total ACNs and metabolome biomarkers were analyzed using a censored regression for panel data with "censReg" and "plm" R-packages [22]. Censored regression models were applied due to the right-skewed distribution of total ACN intake and the considerable proportion of zero values (24% of non-consumers of ACNs). Covariates included in the models were age, sex, and BMI. *p*-values were adjusted for multiple comparisons using the Benjamini–Hochberg method, and adjusted *p*-values <0.05 were considered statistically significant. Second, associations between ACNs from different dietary sources and metabolites were assessed using Mixed Graphical Models (MGM) with the "mgm" R-package [23]. MGMs are undirected probabilistic graphical models able to represent associations between nodes adjusted for all the other variables in the model. MGM specifications were set to allow the maximum number of interactions in the network. Variables in the model were dietary ACN intakes by food categories (8 food groups), and the whole metabolomics set of variables. The agreement between repeated measurements for total dietary intake of ACNs and for ACN intake from different dietary sources was poor across the study evaluations (intra-class correlation coefficient < 0.15). Therefore, all observations of the study were considered independent and were included in MGM analysis (k = 1351). For visual clarity, only the first-order neighborhood of ACNs food sources was plotted.

To evaluate the associations between metabolites and cardiometabolic risk factors linear mixed models were used in random intercepts models adjusted for age, sex, and BMI. Metabolites were selected based on the combination of both analyses, censored regression, and MGM. Standardized coefficients were plotted in a heatmap built using the "pheatmap" R-package (Kolde R (2019). pheatmap: Pretty Heatmaps).

All statistical analyses were performed using R, version 4.1.3. (R foundation, Austria).

3. Results

3.1. Sociodemographic, Clinical, and Dietary Characteristics

At baseline, out of the 720 volunteers who agreed to participate in the study, 624 had completed clinical, dietary, and plasma metabolomics data. Of the 624 participants included, 55% were female, aged (mean ± SD) 45 ± 12 years old, and had a BMI of 25 ± 4 kg/m^2. At 6 months, 380 participants had completed clinical, dietary, and metabolomics data and at

12 months completed data were available for 349 participants. Only, 287 participants had completed clinical dietary and plasma metabolomics data available at all three time points.

The distribution of total ACN intake was right-skewed with a median value of 1.6 (p25–p75: 0.0–26.9) mg/day and a mean value of 26.4 (SD: 60.4) mg/day. Berries were the highest contributors to total ACN intake with a mean contribution of 34%, followed by wines with 33%, and non-alcoholic drinks (which included fruit smoothies and juices) with 20% of the total reported intake. Other fruits (i.e., cherries, apples, and plums) and vegetables were minor contributors with 4% and 2%, respectively. Bakery (pastry, biscuits, and desserts), dairy products (yogurts and strawberry ice creams or ice creams with berries), and other mixed dishes (dishes including vegetables with ACNs) contributed within a similar range between 2 and 3% (Supplementary Table S1).

Participants were divided into tertiles based on the consumed reported intakes of total ACNs as shown in Table 1. There were no significant differences in clinical characteristics across tertiles of ACN intake. Consistently, there were no statistically significant associations between total ACN intake and cardiometabolic risk factors (data not shown). Dietary characteristics are illustrated in Supplementary Table S2. Participants in the highest compared to the ones at the lowest tertile of ACN intake showed statistically significant higher consumption of total protein, saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), alcohol, fruits, and berries.

Table 1. Sociodemographic and clinical characteristics according to tertiles of total ACN intake.

	All ***n*** **= 624** **k = 1351**	**Tertile 1** **<0.3 mg ACN/Day** **k = 453**	**Tertile 2** **0.3–8.9 mg ACN/Day** **k = 448**	**Tertile 3** **>8.9 mg ACN/Day** **k = 450**
Age (years)	44.7 ± 12.3	43.7 ± 12.6	44.2 ±12.5	46.1 ± 11.8
Gender, female (*n*, %)	745 (55)	236 (52)	250 (55)	256 (57)
BMI (kg/m^2)	25 ± 4	25 ± 4	24 ± 3	25 ± 4
WC (cm)	87.5 ± 12.1	88.7 ± 12.4	86.0 ± 11.6	87.7 ± 12
VAT (L)	1.3 (0.7–2.5)	1.5 (0.8–2.6)	1.2 (0.6–2.1)	1.4 (0.8–2.5)
Physical activity (*n*, %)				
Not regular	114 (17)	41 (19)	25 (12)	37 (18)
Once/month last 6 months	52 (89	15 (79	22 (11)	12 (6)
Once/month last 12 months	510 (75)	162 (74)	159 (77)	152 (76)
Smoking status (*n*, %)				
Never	353 (52.2)	114 (52.3)	117 (56.89	97 (48.3)
Former	186 (27.5)	53 (24.3)	53 (25.79)	69 (34.39)
Current	137 (20.3)	51 (23.4)	36 (17.59)	35 (17.4)
SBP (mmHg)	117 ± 16	117 ± 15	116 ± 15	116 ± 16
DBP (mmHg)	81 ± 11	80 ± 10	79 ± 10	80 ± 11
HbA1c (mmol/mol)	34.5 ± 6	34.6 ± 7	33.8 ± 5	34.6 ± 6

Table 1. *Cont.*

	All *n* = 624 k = 1351	Tertile 1 <0.3 mg ACN/Day k = 453	Tertile 2 0.3–8.9 mg ACN/Day k = 448	Tertile 3 >8.9 mg ACN/Day k = 450
TG (mmol/L)	1.1 (0.8–1.6)	1.1 (0.8–1.7)	1.0 (0.8–1.4)	1.1 (0.8–1.6)
TC (mmol/L)	4.9 ± 1.0	4.9 ± 0.9	4.9 ± 0.9	5.1 ± 1.0
HDL (mmol/L)	1.6 ± 0.4	1.5 ± 0.4	1.6 ± 0.4	1.6 ± 0.5
LDL (mmol/L)	3.0 ± 0.9	3.0 ± 0.9	2.9 ± 0.9	3.1 ± 0.9
hsCRP (mg/L)	0.7 (0.3–1.6)	0.8 (0.3–1.6)	0.7 (0.3–1.5)	0.7 (0.3–1.6)

BMI, body mass index; WC, waist circumference; VAT, visceral adipose tissue; SBP, systolic blood pressure, DBP, diastolic blood pressure; HbA1c, hemoglobin A1c; TG, triglycerides; TC, total cholesterol; HDL, high-density lipoproteins; LDL, low-density lipoproteins; hsCRP, high-sensitivity C-reactive protein. Variables following a normal distribution are shown as mean ± SD, and those with a skewed distribution are shown as median (p25–p75).

3.2. Association between Intake of ACN Dietary Sources and Cardiometabolic Risk Factors

Several inverse and direct associations between self-reported intake of ACN-containing food groups and cardiometabolic risk factors were observed (Figure 1). For example, intake of berries, dairy products with berries, and ACN-containing vegetables had inverse associations with visceral adipose tissue volume, while wine had direct associations with total cholesterol, HDL-C, and systolic blood pressure. Other direct associations were found between the intake of berries and hemoglobin A1c, and between ACN-containing drinks with hsCRP.

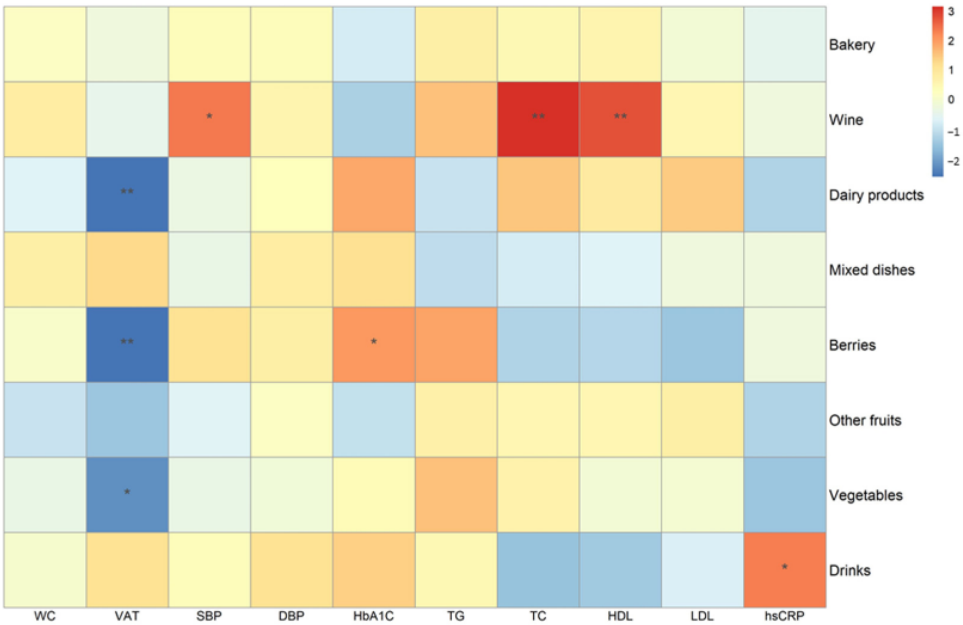

Figure 1. Association between different self-reported food groups and cardiometabolic risk factors in the DCH-NG MAX study (*n* = 624, k = 1351). Standardized coefficients according to linear mixed models with random intercepts adjusting for age, sex, and BMI. * $p < 0.05$, ** $p < 0.01$. *n* = number of subjects, k = total number of observations. TG, triglycerides; SBP, systolic blood pressure; DBP, diastolic blood pressure; WC, waist circumference; HbA1c, hemoglobin A1c; hsCRP, high-sensitivity C-reactive protein; VAT, visceral adipose tissue; TC, total cholesterol; HDL, high-density lipoproteins; TC, total cholesterol; LDL, low-density lipoproteins.

3.3. Metabolome Biomarkers Associated with Total ACN Intake

In censored regression analysis, 10 metabolites were positively associated with total ACN intake (Figure 2). Among them, three were exogenous metabolites, hypaphorine,

salsolinol sulfate, and ethyl glucuronide, two were endogenous metabolites, including linoleoyl carnitine and glycerol, and five were gut microbial metabolites, 4-methylcatechol sulfate, 4′-hydroxy-3′-methoxyphenyl-γ-valerolactone-sulfate (MHPV-S), 5-(4-hydroxy(3,4-dihydroxyphenyl)-valeric acid sulfate (3,4-DHPHVA-S), 3,4-dihydroxyphenylacetic acid sulfate (3,4-DHPA-3S) and indolepropionic acid. On the other hand, only oleoyl carnitine, another endogenous metabolite, was inversely associated with total ACN dietary intake.

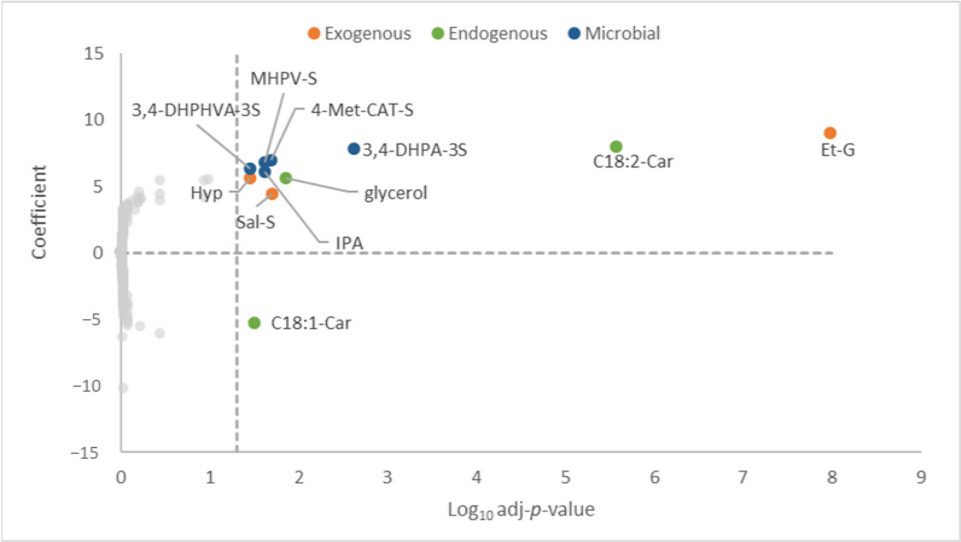

Figure 2. Association between metabolome biomarkers and total intake of ACNs. Censored regression for panel data adjusting for age, sex, and BMI (n = 624, k = 1351). p-values were calculated and adjusted by Benjamini–Hochberg procedure. Adjusted p-values < 0.05 were considered statistically significant. Hyp, hypaphorine; Sal-S, salsolinol sulfate; Et-G, ethyl glucuronide; 4-Met-Cat-S, 4-methylcatechol sulfate; MHPV-S, 4′-hydroxy-3′-methoxyphenyl-γ-valerolactone sulfate; 3,4-DHPHVA-3S, 5-(4-hydroxy(3,4-dihydroxyphenyl)-valeric acid sulfate; 3,4-DHPA-3S, 3,4-dihydroxyphenylacetic acid sulfate; IPA, indolepropionic acid; C18,2-Car, linoleoyl carnitine; C18,1-Car, oleoyl carnitine.

3.4. Metabolome Biomarkers Associated with Intake of ACNs Related to Different ACN Dietary Sources

MGM analysis showed associations between self-reported ACN intake from different dietary sources and 16 metabolites (Figure 3). ACNs derived from dairy products were associated with plasma asparagine, epicatechin sulfate, urolithin C-glucuronide, and acesulfame K. ACNs from the intake of berries were associated with linoleoyl carnitine, salsolinol sulfate, glycochenodeoxycholic-3-sulfate (GCDCA-3S) and 4-methylcatechol sulfate. ACNs from wine consumption were linked with methylpyrogallol sulfate (Met-Pyr-S) and ethyl glucuronide. ACNs from vegetable intake were associated with 2-hydroxybenzoic acid and bergaptol glucuronide. ACNs from other fruits were associated with 3,4-DHPHVA-3S, 5-(3′-hydroxyphenyl)-γ-valerolactone 3′-sulfate (3-HPV-S) and 3,4-dihydroxyphenylacetic acid sulfate (3,4-DHPA-3S). Lastly, the consumption of ACNs from mixed dishes was associated with 1-methylhistidine and 2-hydroxybenzoic acid. Overall, not all the metabolites selected in the MGM analysis were related to ACNs or its microbial metabolites, but to other food components such as acesulfame K, ethyl glucuronide, etc. Therefore, metabolome biomarkers were selected considering both statistical analyses, censored regression, and MGM, to be used for the study of its association with cardiometabolic risk factors.

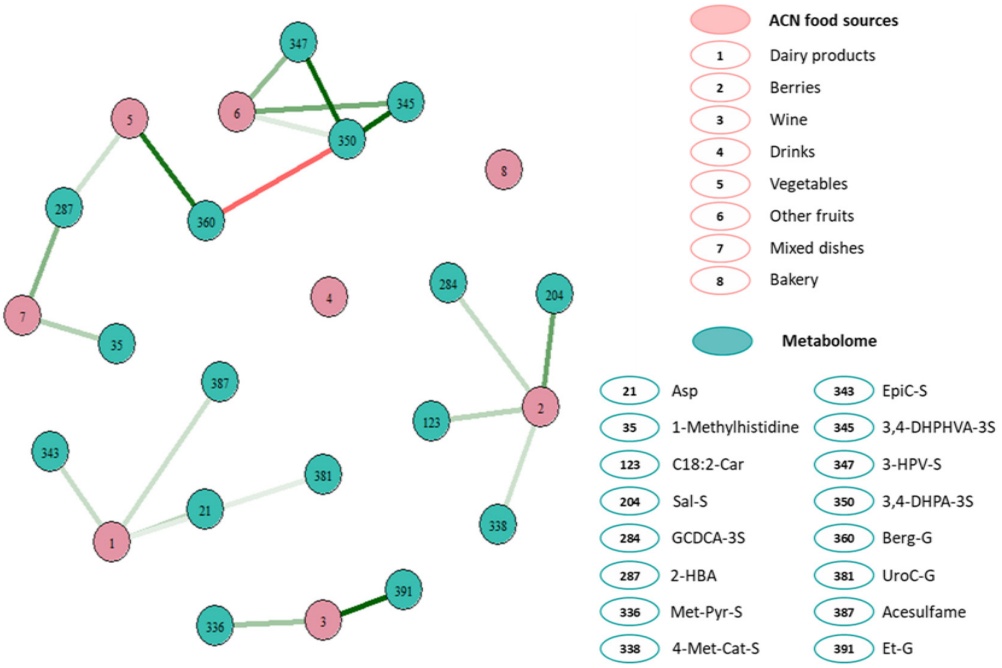

Figure 3. First-order neighborhood of ACNs intake related to different self-reported food groups with plasma metabolome biomarkers according to Mixed Graphical Models in the DCH-NG MAX study (n = 624, k = 1351). Edge intensity reflects the strength of the association from strong direct (dark green) to strong inverse association (dark red). Variables included in the mixed graphical model were ACN intake related to self-reported intake of dairy, berries, wines, non-alcoholic drinks (smoothies and fruit juices), vegetables, other fruits, mixed dishes, and bakery, and all the 408 plasma metabolites quantified with our targeted metabolomics method. n = number of subjects, k = total number of observations. For a detailed list of foods within each category go to Supplementary Table S1. Asp, asparagine; EpiC-S, epicatechin sulfate; UroC-G, urolithin C-glucuronide; GCDCA-3S, glycochenodeoxycholic 3-sulfate; Sal-S, salsolinol sulfate; 4-Met-Cat-S, 4-methylcatechol sulfate; C18:2-Car, linoleoyl carnitine; 2-HBA, 2-hydroxybenzoic acid; Berg-G, bergaptol glucuronide; Met-Pyr-S, methylpyrogallol sulfate; 3-HPV-S, 5-(3′-hydroxyphenyl)-γ-valerolactone 3′-sulfate; 3,4-DHPHVA-S, 5-(4-hydroxy(3,4-dihydroxyphenyl)-valeric acid sulfate; 3,4-DHPA-3S, 3,4-dihydroxyphenylacetic acid sulfate; Et-G, ethyl glucuronide.

3.5. Associations between Selected ACN-Related Metabolome Biomarkers and Cardiometabolic Risk Factors

Metabolites associated with ACN intake in both of the previous analyses were: salsolinol sulfate, 4-methylcatechol sulfate, linoleoyl carnitine, 3,4-DHPHVA-3S, and 3,4-DHPA-S. Figure 4 shows the associations between these metabolites and cardiometabolic risk factors. Out of the metabolites associated with berries' ACNs, salsolinol sulfate and 4-methylcatechol sulfate were inversely associated with visceral adipose tissue volume. In addition, inverse associations were also found between salsolinol sulfate and LDL-C and diastolic blood pressure. Conversely, there was a direct association between salsolinol sulfate and triglyceride levels (Figure 4). Linoleoyl carnitine, 3,4-DHPHVA-3S, 3,4-DHPA-S did not show any statistically significant association with cardiometabolic risk factors.

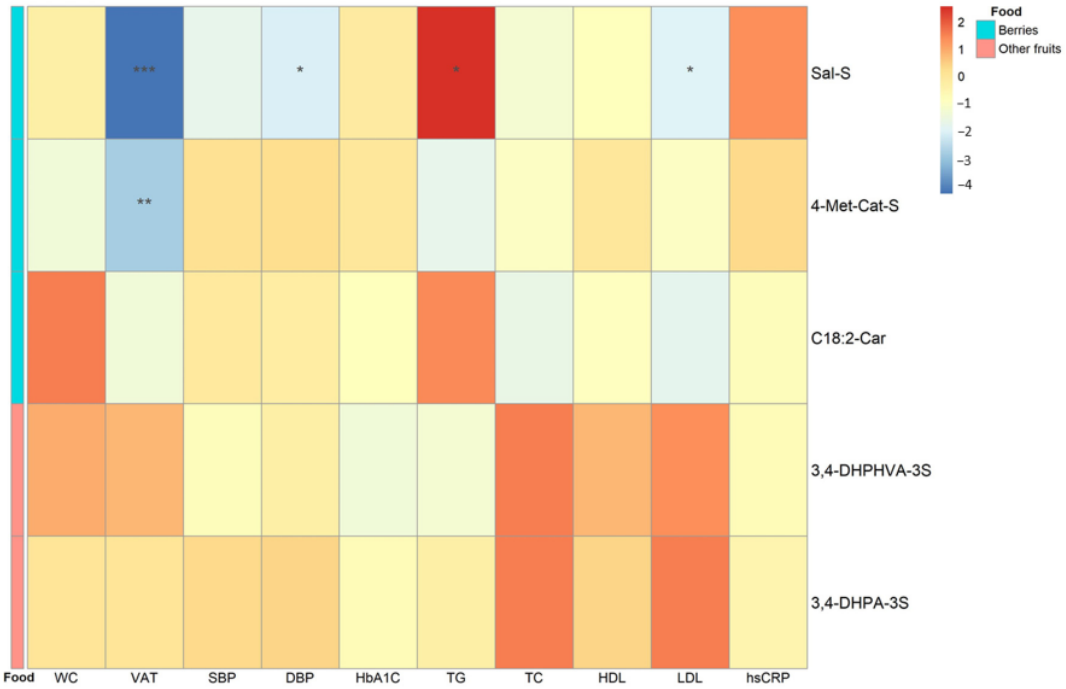

Figure 4. Association between ACN-related selected metabolites and cardiometabolic risk factors in the DCH-NG MAX study (n = 624, k = 1351). Standardized coefficients according to linear mixed models with random intercepts adjusting for age, sex, and BMI. Foods associated with the metabolites according to MGM analysis are displayed by colors in the food column. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. n = number of subjects, k = total number of observations. Sal-S, salsolinol sulfate; 4-Met-Cat-S, 4-methylcatechol sulfate; C18:2-Car, linoleoyl carnitine; 3,4-DHPHVA-3S, 5-(4-hydroxy(3,4-dihydroxyphenyl)-valeric acid sulfate; 3,4-DHPA-3S, 3,4-dihydroxyphenylacetic acid sulfate; TG, triglycerides; SBP, systolic blood pressure; DBP, diastolic blood pressure; WC, waist circumference; HbA1c, hemoglobin A1c; hsCRP, high-sensitivity C-reactive protein; VAT, visceral adipose tissue; TC, total cholesterol; HDL, high-density lipoproteins; TC, total cholesterol; LDL, low-density lipoproteins.

4. Discussion

The present study shows for the first time the specific associations between ACNs related to different dietary sources, and plasma metabolome biomarkers and their association with cardiometabolic risk factors in a free-living population. These results may take into account not only the quantitative and qualitative heterogeneity of ACNs presence in foods but also the internal dose of specific microbial metabolites generated from ACNs which could have been affected by the food matrix. Indeed, food matrices have been shown to influence the microbial metabolism of (poly)phenols [24]. Ultimately, we observed different associations between ACN-related metabolites and cardiometabolic risk factors in relationship with specific foods suggesting a stronger cardiometabolic benefit associated with the consumption of berries.

Up to 80% of the total intake of dietary ACNs came from the consumption of berries, wines, and non-alcoholic drinks in this observational study. Minor contributors were dairy foods, other fruits, and vegetables. While the MGM analysis revealed different metabolomic fingerprints associated with different dietary sources of ACNs, the resultant metabolites were not specific to ACNs. Therefore, we selected metabolites that were also significantly associated with the censored regression analysis. This was a strict criterion but in the context of such low levels of ACN intake in the overall population (median 1.6 mg/day), it is justified. After applying this selection criterion, only metabolites related to ACNs from berries and other fruits (according to MGM) were tested for their association

with cardiometabolic risk factors. Metabolites specifically related to ACNs from other major food sources, such as wines, were excluded. Nonetheless, other studies showed that for example 4-methylcatechol sulfate was increased after a 15-day moderate red wine intervention trial [25]. Therefore, we cannot be fully certain that in our study the same metabolites could be related to other ACN dietary sources. Future randomized controlled trials using single foods are warranted to validate the present results.

Regarding the association between metabolome biomarkers and cardiometabolic risk factors, 4-methylcatechol sulfate showed an inverse association with visceral adipose tissue volume. According to our MGM analysis, 4-methylcatechol sulfate was associated with the intake of ACNs from berries. Similarly, another metabolite associated with ACNs from berries was salsolinol sulfate. Salsolinol sulfate is an alkaloid that has been suggested as a biomarker of banana intake [26]. However, salsolinol can be produced endogenously through dopamine oxidative metabolism [27,28] and may have a role in modulating dopamine neurons activity in the striatum region of the brain [21]. In fact, patients with obesity showed impaired dopamine brain activity, underscoring a potential role for low dopamine activity in obesity (lower reward associated with food intake) [29]. Hence, we speculate that the inverse association between salsolinol sulfate and visceral adipose tissue could be mediated by brain dopamine activity. An animal study showed that a blackberry extract intervention reversed the effects of a high-fat diet increasing dopamine turnover in the brain striatum region [30]. The role of berries on brain dopamine metabolism should be further studied. On the other hand, the other selected metabolites were not associated with cardiometabolic risk factors.

The median value of total ACN intake in the study was 1.6 mg/day, and such intake may not have been high enough to detect metabolome biomarkers found in randomized controlled trials (RCT) with ACN-rich foods [31–33]. Many short or long-term RCTs were conducted with capsulated ACNs or berries to discover biomarkers of ACNs intake. In these trials, daily intakes of ACNs typically varied from 100 to 300 mg as single dose intakes [33,34], or between 50–350 mg/day for four weeks [35–37]. In general, many parent ACNs and up to 70 phenolic compounds resultant of the gut microbial metabolism of ACNs have been identified [35,36]. Even though the majority of these metabolites were not identified in our study, 4-methylcatechol sulfate, 3,4-DHPHVA-3S, and 3,4-DHPA-3S had been previously associated with ACN intake. Maybe, longer half-lives of these metabolites vs. the others, or the competition of polyphenol substrates for bacteria able to metabolize them limited the production of ACNs metabolites under the low levels of ACN intake (exposure) in the study.

Among the strengths of this study are its observational nature and the fact that dietary data were assessed with 24-HDRs instead of food frequency questionnaires. This last characteristic allowed us to have exact intake data both in terms of amounts and specific food items compared to food frequency questionnaires. However, this also brings the limitation of measurement errors in estimating ACN intake and the short time period surveyed (one 24-HDR at each evaluation time). Another limitation was that the median consumption of dietary ACNs within the population of the DCH-NG MAX study was 1.6 mg/day, which was considerably lower than other studies in which the median intake varied between 9.3 to 52.6 mg/day [38–41]. This fact could have limited the number of plasma metabolites associated with dietary ACNs. Furthermore, the mean fasting time of the participants at the time the blood samples were drawn was 5 h and the impact of fasting on serum metabolome is uncertain. Nonetheless, this is the first study evaluating the impact of ACNs coming from different dietary sources on plasma metabolome and therefore our results cannot be contrasted with others. While berries contain other polyphenols in addition to ACNs, further research is needed to fully understand the individual and combined effects of different polyphenols of berries on health outcomes. Our approach to isolating the effects of ACNs from berries was conducted from a bioinformatic approach and a more precise study testing the effects of isolated ACNs from berries should corroborate our results. While berries contain other polyphenols in addition to ACNs, further research

is needed to fully understand the individual and combined effects of different polyphenols of berries on health outcomes. Our approach to isolating the effects of ACNs from berries was conducted from a bioinformatic approach and a more precise study testing the effects of isolated ACNs from berries should corroborate our results. Last, it is not clear if the microbial metabolites were exclusively related to the ACNs from the dietary source pointed out in the MGM analysis or could have been also produced from ACNs coming from other foods, or even from food components other than ACNs (e.g., other polyphenols apart from ACNs). Although MGM models adjust every association for all the other variables included in the analysis, these sources of confounding cannot be ruled out.

In conclusion, this study shows that the metabolomic fingerprint of ACN consumption depended on its dietary sources. Metabolites associated with the consumption of berries' ACNs showed inverse associations with visceral adipose tissue. Future RCTs should validate the importance of these foods for cardiometabolic health and their potential mechanisms of action.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/nu15051208/s1. Table S1. ACN-containing food list in the DCH-NG MAX study, Table S2. Dietary characteristics of the MAX study population according to tertiles of dietary ACN intake.

Author Contributions: Conceptualization: H.M., T.M. and C.A.-L.; data curation: T.M., F.L., A.L.R.-H., J.H. and A.T.; methodology: T.M. and H.M.; resources: C.A.-L., R.L. and J.H.; formal analysis: T.M., H.M., A.M., A.S.-P. and M.C.-C.; software: T.M., A.M., A.S.-P. and M.C.-C.; writing—original draft preparation, H.M. and T.M.; writing—review and editing, N.E.-T., F.L., A.M., R.Z.-R., A.L.R.-H., J.H., A.T., R.L. and C.A.-L.; supervision, R.L., J.H. and C.A.-L. All authors have read and agreed to the published version of the manuscript.

Funding: This work was accomplished as part of the DiGuMet project "Diet × gut microbiome-based metabotypes to determine cardio-metabolic risk and tailor intervention strategies for improved health" supported within the European Joint Programming Initiative "A Healthy Diet for a Healthy Life" (http://www.healthydietforhealthylife.eu), granted by Ministerio de Economía, Industria y Competitividad (MINECO) (Spain, PCIN-2017-076). The study was supported by Grant PID2020-114921RB-C21 and PID2021-128542OA-I00 funded by MCIN/AEI/10.13039/501100011033. The Generalitat de Catalunya's Agency AGAUR of 2021SGR00687. Maria de Maeztu Unit of Excellence grant (CEX2021-001234-M) funded by (MICIN/AEI/FEDER, UE). The work was partially funded from CIBERFES, CB16/10/00269, funded by the Instituto de Salud Carlos III and co-funded by the European Regional Development Fund's "Away to make Europe". The DCH-NG cohort was supported by the Danish Cancer Society, Knæk Cancer 2012 and Den A.P. Møllerske Støttefond (grant nº 10619). The establishment of the DCH-NG MAX study was partly funded by FORMAS (DNR 2016-00314). HM would like to thank the scholarship ajuts de personal investigador predoctoral en formació (APIF) from the University of Barcelona. FL receives support from the Chilean government for doing his PhD through the National Agency for Research and Development (ANID)/Food and Nutrition Doctoral Program/DOCTORADO BECAS CHILE/2019 – 72200061. RZ-R was supported by the "Miguel Servet" program (CPII20/00009) from the Institute of Health Carlos III (co-funded by the European Social Fund (ESF) – ESF investing in your future). C.A-L. is grateful for the ICREA Academia Award 2018. The contributions by RL were supported by Swedish Research Council grant nr 2019-01264 (Discovery and validation of novel biomarkers of gut microbiota, diet and their interactions associated with type 2 diabetes risk).

Institutional Review Board Statement: The DCH-NG cohort study was approved by the Danish Data Protection Agency ((journal number 2013-41-2043/2014-231-0094) and by the Committee on Health Research Ethics for the Capital Region of Denmark (journal number H-15001257).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Data may be available upon request to the Danish Cancer Society (contact: dchdata@cancer.dk).

Acknowledgments: The authors would like to thank all the participants of the study and the staff involved in the DCH-NG MAX study. Myfood24 was developed through Medical Research Council Funding grant G110235. Myfood24 is now being supported by spinout company Dietary Assessment Ltd. Request to use myfood24 should be made to enquiries@myfood24.org. The software for the assessment of polyphenol intake from food surveys (UBTT0422) (http://diposit.ub.edu/dspace/handle/2445/187698) (7 February 2023) was developed through the institutional support of the University of Barcelona, the Bellvitge Biomedical Research Institute (IDIBELL) and the Centro de Investigación Biomédica en Red (CIBER—Instituto de Salud Carlos III). Requests to use this software should be made to candres@ub.edu or rzamora@idibell.cat.

Conflicts of Interest: The authors declare no conflict of interest.

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Supplementary Table S1: ACN-containing food list in the DCH-NG MAX study	
food_name	**food_category**
Alpro Blueberry Yogurt Alternative	drinks
Apple crumble	Refined cereals
Apple dried	Other fruits
Apple jelly	Sugar jam syrups
Apple, raw	Other fruits
Apples, cooking, baked with sugar, flesh only, weighed with skin	Other fruits
Apples, cooking, stewed with sugar, flesh only	Other fruits
Apples, cooking, stewed without sugar, flesh only	Other fruits
Aubergine, stuffed with vegetables, cheese topping	Vegetables
Bagel sandwich w/ salami philadelphia cheese green salad and olives	mixed dishes
Bean loaf, mixed beans, homemade	Vegetables
Beanburger, aduki, fried in rapeseed oil, homemade	Vegetables
Beanburger, red kidney bean, fried in rapeseed oil	Vegetables
Beef bourguignon, homemade	mixed dishes
Beef bourguignonne, made with lean beef, homemade	mixed dishes
Beef, mince, with vegetables, stewed	mixed dishes
Biscuit, coarse grain with fruit and vegetables	Refined cereals
Biscuit, sweet	Refined cereals
Black currant, jam	berry
Black currant, marmalade	berry
Blackberry, frozen, unsweetened	berry
Blackberry, jam	berry
Blackberry, raw	berry
Blueberries, raw	berry
Blueberry, frozen, unsweetened	berry
Bouillabaisse, homemade	mixed dishes
Breakfast cereal, müsli	Cereals wholegrain
Buttermilk dessert	Dairy
Cape gooseberries, raw	berry
Carrot juice, canned	Vegetables
Carte D'Or Strawberry Ice Cream	Dairy
Casserole, bean and mixed vegetable, homemade	Vegetables
Cheeseburger, salad and dressing, fast food	mixed dishes
Cheesecake, fruit, individual	Refined cereals
Cheesecake, homemade	Refined cereals
Cherries, stewed with sugar	Sauces dips dressings
Cherry, canned in sugar, jam	Sugar jam syrups
Cherry, raw	Other fruits
Chicken chasseur, homemade, weighed with bone	mixed dishes
Chilli con carne, homemade	mixed dishes
Chocolate, fancy and filled	Chocolate
Cookie, truffle product / unbaked, industrially produced	Refined cereals
Coq au vin, homemade, weighed with bone	mixed dishes
Cream pastry	Refined cereals
Cream pastry, strawberry cake	Refined cereals
Currant, black, raw	berry
Currant, red, raw	berry
Curry mayonnaise salad	Sauces dips dressings

Curry, chicken korma, homemade	mixed dishes
Curry, chicken, made with cookin sauce	mixed dishes
Curry, fish and vegetable, Bangladeshi, homemade	mixed dishes
Curry, lamb, made with cookin sauce	mixed dishes
Danish pastry, christmas cake	Refined cereals
Doughnuts, with jam	Refined cereals
Duck a l'orange, excluding fat and skin, homemade	mixed dishes
Florette Mixed Salad	mixed dishes
Fruit paste	Other fruits
Fruit sauce for ice cream Ohoj	berry
Grape, raw	berry
Guacamole, homemade	Sauces dips dressings
Hamburger and bacon, salad and dressing, fast food	mixed dishes
Hamburger, bacon and cheese, salad and dressing, fast food	mixed dishes
Hamburger, big patty, salad and dressing, fast food	mixed dishes
Hamburger, chicken, salad and dressing, fast food	mixed dishes
Hamburger, medium patty, salad and dressing, fast food	mixed dishes
Hamburger, small patty, salad and dressing, fast food	mixed dishes
Hellmann's Honey & Mustard Dressing	Sauces dips dressings
Herring, pickled, in madeira sauce, canned	mixed dishes
Ice cream, dairy, popsickle, strawberry	Dairy
Innocent Pure Fruit Smoothie - Kiwis, Apples and Limes	drinks
Innocent Pure Fruit Smoothie Blackberries, Strawberries & Blackcurrants	drinks
Innocent Pure Fruit Smoothie Mangoes & Passion Fruits	drinks
Innocent Pure Fruit Smoothie Strawberries & Banana	drinks
Jam, all types	berry
Kellogg's Special K Red Berries	Refined cereals
KETTLE Chips Mature Cheddar & Red Onion	Refined cereals
Knorr Garde d'Or Bearnaise Sauce	Sauces dips dressings
Knorr Garde d'Or Hollandaise Sauce	Sauces dips dressings
Lactofree Lactose Free Real Fruit Yogurts Strawberry & Raspberry	Dairy
Lasagne, homemade	mixed dishes
Lasagne, homemade, with extra lean minced beef	mixed dishes
Lasagne, vegetable, wholemeal, homemade	Vegetables
Lentil and nut roast, homemade	Vegetables
Lettuce, looseleaf, raw	mixed dishes
Lingonberry, jam	Berry
Marmalade, all types	berry
Milkshake	Dairy
Mushrooms in white sauce	Sauces dips dressings
Nectarine, raw	Other fruits
Olives, black, ripe, salted, oil-coated,	Other fruits
Olives, black, without stone, in brine	Other fruits
Onion red	mixed dishes
Onions, baked	mixed dishes
Paella	mixed dishes
Pastry, frangipani pastry	Refined cereals
Pita sandwich with chicken, salad and dressing, fast food	mixed dishes
Pizza with vegetables, fruit, tomato and cheese, fast food	Vegetables
Plum, raw	Other fruits

Plums, average, stewed with sugar, flesh and skin	Other fruits
Port	Wine
Rasberry, raw	berry
Raspberries, stewed with sugar	berry
Raspberries, stewed without sugar	berry
Raspberry, frozen	berry
Raspberry, marmalade	berry
Ratatouille, homemade	Vegetables
Red wine sauce ready-made sauce fat 1,5% Spisa Rydbergs	Sauces dips dressings
Risotto, chicken, homemade	mixed dishes
Risotto, plain, homemade	Vegetables
Risotto, vegetable, brown rice	Vegetables
Risotto, white rice, vegetable, homemade	Vegetables
Rose hip, dried, powder	berry
Salad dressing, oil and vinegar	Sauces dips dressings
Salad, Greek	Vegetables
Salad, tomato and onion	Vegetables
Salad, Waldorf	Vegetables
Scrabed raw beef	mixed dishes
Sorbet, fruit	Dairy
Squash, added sugar, ready-to-drink	Sugar jam syrups
Squash, currant, black, sweet, ready-to-drink	drinks
Squash, elderberry, sweet, ready-to-drink	drinks
Squash, no added sugar, ready-to-drink	drinks
Squash, sweet, ready-to-drink	drinks
Stir fry vegetables w/ chicken noodles	mixed dishes
Stir fry vegetables w/ noodles	Vegetables
Stir-fry vegetable mix Classic woked in rapeseed oil	Vegetables
Strawberries, frozen, unsweetened	berry
Strawberry, jam	berry
Strawberry, marmalade	berry
Strawberry, raw	berry
Swiss roll, all types	Refined cereals
Swiss roll, raspberry stuffing	Refined cereals
Tropical juice ready to drink	drinks
Veal shin casserole Osso buco	mixed dishes
Venison in red wine and port, homemade	mixed dishes
Waffles plain or egg waffles	Refined cereals
Wine, red	Wine
Wine, red, sweet	Wine
Wine, white	Wine
Wine, white, dry	Wine
Wine, white, medium	Wine
Wine, white, Rhine wine	Wine
Yoghurt with peach melba, 3.2% fat	Dairy
Yoghurt, strawberries, 3.1% fat	Dairy
Yoghurt, with fruit,1.5% fat	Dairy
Yogurt, with fruit, 0.1 % fat, no sugar added	Dairy
Yogurt, with fruit, 0.4 % fat	Dairy

Supplementary Table S2. Dietary characteristics of the MAX study population according to tertiles of dietary ACN intake.

	All n= 624 k=1,353	Tertile 1 <0.29 mg ACN/day	Tertile 2 0.29-8.9 mg ACN/day	Tertile 3 >8.9 mg ACN/day
Dietary characteristics				
Energy (10^3 kcal/d)***	2.1 ±0.8	1.9 ±0.8	2.1 ±0.8	2.2 ±0.8
SFA (g/d)*	27 ±15	25 ±15	28 ±15	28 ±16
MUFA (g/d)***	29 ±16	27 ±16	31 ±16	30 ±16
PUFA (g/d)**	14 ±7	13 ±8	15 ±7	14 ±7
Total sugars (g/d)***	73 ±50	58 ±42	77 ±46	73 ±50
Sucrose (g/d)*	35 ±32	31 ±31	37 ±34	37 ±35
Protein (g/d)*	88 ±40	83 ±39	88 ±36	91 ±44
Dietary fiber (g/d)**	22 ± 11	20 ± 11	24 ± 11	22 ± 11
Alcohol (g/d)***	0 (0 - 13.2)	0 (0-0)	0 (0-9.3)	9.5 (0-28.5)
Sodium (g/d)	3.04 ± 1.7	2.96 ±1.8	3.09 ±1.6	3.03 ±1.7
Food intake				
Cereal whole grain (g/d)	115 (45-200)	105 (35-205)	125 (60-213)	110 (45-190)
Cereal refined (g/d)	0 (0-80)?	0 (0-90)	0 (0-80)	0 (0-61)
Fruits (g/d)***	150 (0-313)	0 (0-120)	150 (0-250)	110 (0-229)
Berries (g/d)***	0 (0-10)	0 (0-0)	0 (0-6.5)	10 (0-35)
Vegetables (g/d)	140 (30-290)	190 (60-360)	210 (60-394)	247 (98-400)
Legumes (g/d)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)
Nuts and seeds (g/d)	0 (0-10)	0 (0-1.5)	0 (0-15)	0 (0-15)

SFA: Saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids. *****p for trend<0.05, ** p for trend<0.01, *** p for trend<0.001 using age- and sex-adjusted generalized linear models. Variables with skewed distribution were log-transformed before entering the analyses.

GENERAL DISCUSSION

6. GENERAL DISCUSSION

This chapter provides an overall discussion of the main results presented earlier.

ACNs are intensively metabolized by gut microbiota. Therefore, in the systematic review we support the suggestion for a multi-metabolite panel, including several gut bacterial metabolites as biomarkers of intake for blueberries and cranberries. Similar, multi-metabolite panels were found as biomarkers of intake for raspberries, strawberries, blackcurrants, and blackberries. As there were not specific biomarkers for individual berries more studies are needed to validate multi-biomarker panels for improving the estimation of berry intake using metabolites concentration in biological samples.

In line with this need for further metabolomics analyses, in publications from the ATTACH study, we showed that six parent ACNs were significantly altered in urine and three in feces after the intake of the ACN-rich juice. Conversely, in plasma we couldn't detect differences in parent ACNs. This can be explained by two factors; the time of blood sampling because plasma was collected at baseline and at 6h after ACNs intake on day 28. As mentioned earlier and according to the literature, parent ACNs can reach their C_{max} between approximately 30 minutes and 2h, and then they start to be cleared from circulation (Krga and Milenkovic 2019; Fang 2014). Therefore, it is believed that there were no parent ACNs detected in the plasma in publication 2 because of blood sampling time. Nonetheless, three plasma metabolites were significantly associated with ACNs intake in the ATTACH study, namely 2,4,6-trihydroxybenzaldehyde (THBAld), 4'-hydroxy-3'-methoxyphenyl-γ-valerolactone glucuronide (MHPV-G) and 3',4'-dihydroxyphenyl-γ-valerolactone (3,4-DHPV). Other gut bacterial fermentation products altered by the intervention in urine were o-coumaric acid (oCOU), 3′ -methylepicatechin sulphate (MeEC-3-S), 2-hydroxybenzoic acid-sulphate, 3,4-DHPV-3-glucuronide, and 3,4-DHPV-4-glucuronide.

Regarding, the effects on cancer cell migration, metabolites extracted from plasma after a 28-day intervention with an ACN-rich juice were able to prevent the migration of the human colon carcinoma cell line HT-29 in an in-vitro model. Furthermore, plasma extracts enhanced the anti-migratory effect of 5-Fluorouracil (5-FU) in comparison to the use of 5-FU alone. However, the plasma metabolites responsible for this effect were not analyzed in publication

2. In publication 3, PAMs were able to inhibit the migration and the expression of CAMs in PANC-1 and endothelial cells by decreasing the phosphorylation of NF-kB p65 and FAK. In addition, publication 3 described the associations of these metabolites with the reduction of pancreatic cancer cells migration. Only urinary oCOU and peo-3-gal were inversely associated with the reduction of PANC-1 migration. Interestingly, oCOU has showed anti-cancer activities on human breast cancer cells as indicated by Sen et al. (Sen et al. 2013). On the other hand, peonidin-glucosides were the most abundant ACN in the juice (see table 4.1). The novelty of this work was that it was the first one to examine the effect of ACNs at a physiological concentration on cancer cells in an in-vitro model after the consumption of ACNs. Previously, some in-vitro models were used to investigate the anti-cancer effect of ACNs on cancer cells; however, ACNs were not retrieved from biological samples following the consumption of ACNs. Surprisingly, levels of plasma metabolites significantly altered by the ACN-rich juice were not associated with the reduction of PANC-1 migration, despite prior in-vitro evidence showing that high doses of γ-valerolactones had anti-cancer effects (Forester et al. 2014; Sankaranarayanan et al. 2019; Augusti et al. 2021). The fact that urinary metabolites but not the plasma ones were associated with the results in cancer cells is intriguing and argues for the influence of intra-individual factors affecting ACN metabolism and bioavailability. A higher urinary output of ACNs and their metabolites could be the result of higher absorption or different gut microbiota metabolism. Another possible explanation could be that although the protein precipitation method has been proven to be an effective pre-processing step for detecting and quantifying many gut microbiota derivatives including valerolactones in plasma (González-Domínguez et al. 2020), some metabolites may remain undetected, particularly if they were present at very low concentrations. Nevertheless, we believe that these results opened a window for future studies to validate the anti-migration effects of these and other ACN-derived metabolites.

The results from the DCH NG-MAX study showed for the first time the specific associations between ACNs related to different dietary sources, and plasma metabolome biomarkers and their association with cardiometabolic risk factors in a free-living population. These results may took into account not only the quantitative and qualitative heterogeneity of ACNs presence in foods, but also the internal dose of specific microbial metabolites generated from ACNs which could have been affected by food matrix. Indeed, food matrices have been

shown to influence the microbial metabolism of (poly)phenols (Roura et al. 2008). Ultimately, we observed different associations between ACN-related metabolites and cardiometabolic risk factors in relationship with specific foods suggesting a stronger cardiometabolic benefit associated with consumption of berries. Up to 80% of the total intake of dietary ACNs in this Danish population came from the consumption of berries, wines, and non-alcoholic drinks. Minor contributors were dairy foods, other fruits, and vegetables. While the MGM analysis revealed different metabolomic fingerprints associated with different dietary sources of ACNs, the resultant metabolites were not specific to ACNs. Therefore, we selected metabolites using a double criteria combining the results from two different statistical analyses. This was a strict criterion but in the context of such low levels of ACN intake in the overall population (median 1.6 mg/day), it is justified. After applying this selection criterion, only metabolites related with ACNs from berries and other fruits (according to MGM) were tested for their association with cardiometabolic risk factors. At the end, we showed that 4-methylcatechol-sulfate showed an inverse association with visceral adipose tissue volume. According to our MGM analysis, 4-methylcatechol-sulfate was associated with intake of ACNs from berries. Similarly, another metabolite associated with ACNs from berries was salsolinol-sulfate. Salsolinol-sulfate is an alkaloid which has been suggested as a biomarker of banana intake (M. Ulaszewska et al. 2020). However, salsolinol can be produced endogenously through dopamine oxidative metabolism (Faraj et al. 1989; Rojkovicova et al. 2008) and may have a role on modulating dopamine neurons activity of the striatum region of the brain (Xie and Ye 2013). Future randomized controlled trials using single foods are warranted to validate the present results.

The link between the ATTACH and the DCH-NG MAX studies is that valerolactones were associated with ACNs consumption in both studies. This is interesting as it showed that regardless of how low the concentration of ACNs intake might be, these gut-related metabolites are detectable in plasma and urine. Valerolactones have been suggested to have beneficial effects on various health outcomes, particularly cardiometabolic diseases and cancer. For example, Ana Rodriguez-Mateos et al, showed in an RCT that valerolactones after cranberry intake were associated with an increase in FMD, sign of improved vascular function (Rodriguez-Mateos et al. 2016). On the other hand, Josep Rubert et al, described that long-term exposure to valerolactones may reduce colorectal cancer propagation by

decreasing the spheroid size of 3D HCT116 spheroids (Rubert et al. 2022). These results come in line with the main objectives and results of this thesis, supporting a role for ACN-derived microbial metabolites in prevention of cancer cells migration. Valerolactones may be interesting metabolites for future research because they remain large times in circulation (Di Pede et al. 2022). Nonetheless, it is important to mention that plasma valerolactones can come also from flavan-3-ols, such as proanthocyanidins, apart from ACNs (Mena et al. 2019). Proanthocyanidins have been reported to be abundant in berries, cacao products, nuts and tea leaves (Rauf et al. 2019; Knaze et al. 2012; Hellström, Törrönen, and Mattila 2009). In this regard, plasma valerolactones levels increased after three months of green tea supplementation as revealed by Clarke KA et al, (Clarke et al. 2016). Daily intake of proanthocyanidins vary as reported in some cohorts studies. For example, in DCH-NG MAX study in Danish population, the mean intake of proanthocyanidins was 232 mg/day (Lanuza, Zamora-Ros, Rostgaard-Hansen, Tjønneland, et al. 2022), while in a study in U.S. population estimated a mean intake of 57.7 mg/day for persons >2 years old (Gu et al. 2004). In the EPIC study (Knaze et al. 2012), the mean intake of proanthocyanidins was 217.2 mg/day in the Mediterranean countries, 177.9 mg/day in the non-Mediterranean countries, and 198.4 mg/day in the UK. It's challengeable to confirm whether ACNs or proanthocyanidins were the source of the valerolactones in the biological samples after the consumption of foods rich in both of them in DCH-NG MAX study. However, it is believed that ACNs were the source of valerolactones in plasma in ATTACH study, as they were significantly different from the control group who consumed an ACN-depleted control drink.

One of the main differences between the ATTACH (publications 1 and 2) and the DCH-NG MAX study (publication 3) was the concentration of ACNs consumed in both studies. In publications 1 and 2, participants consumed approximately 311 mg/day of ACNs in the form of juice for 28 days while following a low-polyphenol diet during the intervention. On the other hand, in publication 3, participants consumed a low amount of ACNs (median 1.6 mg/day, mean 26.4 mg/day) which was part of their habitual diet. The observational design of the DCH-NG MAX gave us information about the participants' habitual diets and allowed us to demonstrate how the food source for dietary ACNs affected the plasma metabolic fingerprint. This was made possible in part because 24h dietary recalls were used to gather dietary information rather than food frequency questionnaires. However, the short time

period for dietary measurement was a limitation though (one 24h dietary recall at each evaluation time) to have a representative measurement of habitual ACNs consumption. In comparison to other cohort studies, the daily median intake of dietary ACNs among the participants in the DCH-NG MAX study was 1.6 mg, which was lower than other studies (between 9.3 to 52.6 mg/day) (Zamora-Ros et al. 2011; Lagiou et al. 2008; Rossi et al. 2007). The amount of plasma metabolites linked to dietary ACNs may have been constrained as a result of this fact. However, since this was the first study that examined the effects of ACNs from various dietary sources on the plasma metabolome, our results cannot be compared to those of other studies.

CONCLUSIONS

7. CONCLUSIONS

From the results obtained from the different studies presented in this thesis derived the following conclusions:

1. The consumption of dietary ACNs led to significant changes of metabolites produced by gut bacterial fermentation of ACNs in plasma, urine, and feces of healthy individuals. Even at low doses of ACNs, like the ones in the observational study, plasma valerolactones were significantly associated with the intake of ACN-rich foods. Combined with it's long half-life in circulation and in-vitro and in-vivo evidences for a positive health effect, future studies centered in valerolactones properties are warranted. Indeed, multi-metabolite panels suggested in our review included valerolactones as biomarkers of berries intake.

2. ACNs and their metabolites isolated from plasma after a 28-day intervention with dietary ACNs showed inhibitory effects on colon and pancreatic cancer cell migration, potentially through the modulation of CAMs. Such activity was related with lower ROS and inhibition of the NF-kB and FAK pathways. These findings suggest that ACNs could have a potential role in cancer prevention and treatment.

3. Although plasma levels of gut metabolites affected by an ACN-rich juice intervention were not associated with the inhibition of cancer cell migration of plasma extracts, urinary metabolites did. Urine samples were collected during 24-h and therefore, adherence or individual factors could be related with the anti-migratory effects of the plasma extracts. Elucidation of these individual factors is critical for exploitation of dietary ACNs in cancer prevention and treatment strategies.

4. The results of the DCH-NG MAX study suggest that dietary sources of ACNs are associated with specific plasma metabolomic profiles, and that berries among all the dietary sources of ACNs could have the most significant impact on cardiometabolic health in a habitual diet. These findings could have important implications for the

development of personalized nutrition interventions for the prevention and management of cardiometabolic diseases.

5. As a general conclusion, dietary ACNs are intensively metabolized by gut microbiota leading to the production of several metabolites with longer half-lives, mainly γ-valerolactones, which can be altered even at the low doses of consumption. Therefore, the main conclusion of this thesis is that increasing the dietary intake of ACNs may become essential for improving human health.

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ANNEX

9. ANNEX

9.1 Other publications

This annex includes other publications that are not part from the thesis.

- **Publication 1:** A mixture of four dietary fibres ameliorates adiposity and improves metabolic profile and intestinal health in cafeteria-fed obese rats: an integrative multi-omics approach. Núria Estanyol-Torres, Cristina Domenech-Cocac, Raúl González-Domínguez, Antonio Miñarro, Ferran Reverter, Jose Antonio Moreno-Muñoz, Jesús Jiménez, Manel Martín-Palomas, Pol Castellano-Escuder, **Hamza Mostafa**, Santi García-Vallvé, Nerea Abasolo, Miguel A. Rodríguez, Helena Torrell, Josep M del Bas, Alex Sanchez-Pla, Antoni Caimari, Anna Mas-Capdevila, Cristina Andres-Lacueva, Anna Crescenti. *The Journal of Nutritional Biochemistry*. 111 (2023) 109184; https://doi.org/10.1016/j.jnutbio.2022.109184

  Journal impact factor: 6.117
  Journal quartile: Q1 (Category: Nutrition & Dietetics, 13/109)

Available online at www.sciencedirect.com

ScienceDirect

Journal of Nutritional Biochemistry 111 (2023) 109184

Journal of Nutritional Biochemistry

RESEARCH PAPER

A mixture of four dietary fibres ameliorates adiposity and improves metabolic profile and intestinal health in cafeteria-fed obese rats: an integrative multi-omics approach

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Received 30 August 2021; received in revised form 17 May 2022; accepted 15 September 2022

Abstract

The aim of this study was to assess the effects of a mixture of four dietary fibers on obese rats. Four groups of male Wistar rats were fed with either standard chow (STD) or cafeteria diet (CAF) and were orally supplemented with either fibre mixture (2 g kg^{-1} of body weight) (STD+F or CAF+F groups) or vehicle (STD+VH or CAF+VH groups). We studied a wide number of biometric, biochemical, transcriptomic, metagenomic and metabolomic variables and applied an integrative multivariate approach based on multiple factor analysis and Pearson's correlation analysis. A significant reduction in body weight, adiposity, HbA1c and HDL-cholesterol serum levels, and colon MPO activity was observed, whereas cecal weight and small intestine length:weight ratio were significantly increased in F-treated groups compared to control animals. CAF+F rats displayed a significant enhancement in energy expenditure, fat oxidation and fresh stool weight, and a significant reduction in adiponectin and LPS serum levels, compared to control group. Animals in STD+F group showed reduced serum LDL-cholesterol levels and a significant reduction in total cholesterol levels in the liver compared to STF+VH group. The intervention effect was reflected at the metabolomic (*i.e.*, production of short-chain fatty acids, phenolic acids, and amino acids), metagenomic (*i.e.*, modulation of Ruminococcus and *Lactobacillus* genus) and transcriptomic (*i.e.*, expression of tight junctions and proteolysis) levels. Altogether, our integrative multi-omics approach highlights the potential of supplementation with a mixture of fibers to ameliorate the impairments triggered by obesity in terms of adiposity, metabolic profile, and intestinal health.

Keywords: Dietary fiber; diet-induced obesity; multi-omics; intestinal health; metabolic syndrome; adiposity.

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https://doi.org/10.1016/j.jnutbio.2022.109184

1. Introduction

Obesity is defined as an abnormal or excessive body fat accumulation that may impair health and is considered a major risk factor for many chronic diseases, including metabolic syndrome (MetS). MetS is a pathologic condition composed of a constellation of risk factors including central obesity, insulin resistance, systemic hypertension, and atherogenic dyslipidemia. These interrelated conditions can lead to a prothrombotic and pro-inflammatory state, commonly associated with accelerated atherosclerosis and increased risk of cardiovascular disease, among others. Among noncommunicable diseases, MetS and obesity are major health hazards worldwide and leading causes of mortality and morbidity [1,2].

Obesogenic high-calorie, low-fiber diets are considered one of the principal risk factors for obesity and MetS. Therefore, diet is among the most important modifiable behaviors for the prevention and amelioration of these multifactorial disorders. Indeed, dietary fiber intake is suggested to prevent diet-induced obesity and to lower the risk of MetS [3,4], having a direct impact on insulin resistance, glucose tolerance and weight control, while serving as a rich source of antioxidant and anti-inflammatory nutrients [5]. Moreover, fiber is well known to promote healthy gut microbiota composition, which can in turn modulate the host response to diet [3]. However, the available evidence suggests that dietary fiber consumption is associated only with small improvements in body weight, and the effects in terms of changes in body composition and fat mass are not conclusive [6]. Given their main properties (*i.e.*, viscosity, water solubility and fermentation rate), dietary fibers can be classified into different groups. For instance, inulin and resistant starch are defined as nonviscous, soluble, and fermentable fibers, whereas guar gum is a viscous, soluble and fermentable fiber. These properties determine the health effects of dietary fiber through its ability to be fermented by the intestinal microbiota and produce a prebiotic effect, to induce mechanical and laxative effects and, therefore, to regulate digestive function, and to reduce nutrient absorption in the intestine [7].

In such multifactorial disorders as obesity and MetS, it is fundamental to combine multidimensional data to get a comprehensive picture of the effects elicited by dietary interventions, describing intrinsic factors (*e.g.*, genetics, gut microbiome), extrinsic factors (*e.g.*, diet) and their interconnections. The combination of multi-omics techniques, therefore, is a powerful strategy for broadening the current understanding of MetS alterations and the beneficial effects of these treatments.

The aim of this work was to evaluate the effect of an intervention with a fiber mixture comprising 36% inulin, 31.5% hydrolyzed guar gum, 22.5% resistant maltodextrin, and 10% dehydrated plum on an obese metabolic phenotype. To this end, rats with diet-induced obesity [8] were fed with a dose of fiber equivalent to human daily consumption [9]. The effects produced by the treatment were widely investigated, taking into consideration biometric, biochemical, transcriptomic, metagenomic, and metabolomic data, and using integrative multivariate statistical techniques.

2. Materials and methods

2.1. Animals, diets, and treatments

The Animal Ethics Committee of Rovira i Virgili University (Tarragona, Spain) and the *Generalitat de Catalunya* approved all the procedures (reference number 9823 of the Generalitat de Catalunya). The experimental protocol adhered to the "principles of laboratory animal care" and was carried out in accordance with the European Communities Council Directive (86/609/CEE).

The animals used in this study were 4-week-old male Wistar rats (Envigo, Barcelona, Spain) housed singly at 22 °C for a light/dark period of 12 h (lights on at 8 AM) and with free access to food and water. All animals were subjected to a 4-day adaptation period in which they voluntary consumed 200 μL of diluted low-fat condensed milk from a feeding syringe. After that, the rats were randomly distributed into four experimental groups (n=10) depending on the diet and the oral treatment received over 8 weeks: the STD+VH group was fed with a standard chow diet (STD, 2018 Teklad Global 18% Protein Rodent Diet, 3.1 kcal g^{-1}; Envigo, Barcelona, Spain) supplemented daily with 2 g kg^{-1} of body weight of maltodextrin dissolved in diluted low-fat condensed milk, which was diluted 1:4 with water (vehicle, VH); the STD+F group was fed with STD supplemented daily with 2 g kg^{-1} of body weight of a combination of fibers (F) dissolved in diluted low-fat condensed milk; the CAF+VH group was fed with an obesogenic cafeteria diet (CAF) supplemented daily with 2 g kg^{-1} of body weight of maltodextrin dissolved in diluted low-fat condensed milk; and the CAF+F group was fed with CAF supplemented daily with 2 g kg^{-1} of body weight of the F dissolved in diluted low-fat condensed milk. The CAF diet was prepared as previously described [8]. The F treatment consisted of a mixture of four different natural components, comprising 36% inulin, 31.5% hydrolyzed guar gum, 22.5% resistant maltodextrin, and 10% dehydrated plum (Laboratorios Ordesa S.L., Barcelona, Spain). This treatment contained a total of 78% of fibers, of which 75% were fermentable, while the remaining 22% was mainly composed of other carbohydrates (*e.g.*, glucose, sucrose). Given an average rat's weight of 270 g, the dose of 2 g kg^{-1} day^{-1} of the F is equivalent to the daily consumption of 22.4 g of fiber by a 60 kg human [9]. In most countries, the recommended daily intake of dietary fiber is 25–35 g [10], so the dose of F used in this study can be considered acceptable for human consumption.

The body weight and food intake data were recorded once a week. In the seventh week of the study, the animals were housed in metabolic cages (3700M071 model for rats from 150 g to 300 g, Panlab, Barcelona, Spain) to collect urine for a period of 24-h in fasting conditions. Urine samples were stored at -80 °C in tubes containing 270 mg of boric acid. In the eighth week of the study, rats were fasted for seven hours and were subjected to an oral glucose tolerance test (OGTT) following a previously described procedure [11].

After eight weeks, the animals were deprived of food for 6 hours (from 8 h to 14 h), anaesthetized with sodium pentobarbital (Merck Life Science, Madrid, Spain), and blood was collected through cardiac puncture. Serum and plasma were obtained from blood samples by centrifugation and were stored at -80°C until further analysis. PBMCs were obtained as previously described [12] and PBMC pellets were stored at -80 °C until obtaining RNA. White adipose tissue depots (retroperitoneal-RWAT, inguinal-IWAT, mesenteric-MWAT and epididymal-EWAT), the liver, small intestine, colon, and cecum (with the contents) were rapidly removed after death, weighed, frozen in liquid nitrogen and stored at -80 °C until further analysis. The length of the small intestine and colon were recorded as measures related to inflammation.

2.2. Serum, plasma, and tissue biochemical analysis

Biochemical analyses of serum, liver, and colon samples were performed using the methodology described in the Supplementary Material file. The homeostasis model assessment-estimated insulin resistance (HOMA-IR) index was calculated following the formula proposed by Matthews [13]. Additionally, the insulin sensitivity was assessed by the revised quantitative insulin sensitivity

check index (R-QUICKI), using the formula described by Perseghin [14].

2.3. Adiposity

The adiposity level of each animal was determined via an adiposity index computed as the sum of the RWAT, IWAT, MWAT, and EWAT depot weights or, in the case of the visceral adiposity index, the sum of the RWAT, IWAT, and EWAT depot weights, and expressed as a percentage of total body weight.

2.4. Body composition analysis

Lean, fat and total water measurements (in percentage of body weight) were performed three times for each animal during the study – at baseline, at 4 weeks and before sacrifice – by quantitative magnetic resonance using an EchoMRI-700 device (Echo Medical Systems, L.L.C., Houston, TX, USA) [11,13,14].

2.5. Indirect calorimetry and locomotor activity

The OxyletPro system (Panlab, Barcelona, Spain) was used to evaluate the animals' physical activity and respiratory metabolism. The measurements were performed during weeks five and six of the study for 23 h (from 9 h to 8 h of the following day). Data collected during the first 4 h were discarded, established as an acclimatation period, which finally corresponded to a record of 19 h per animal. During the first few hours (from 12 h to 17 h), the animals were left with water but without food to obtain the basal metabolism values (fasting; basal oxidation of carbohydrates and lipids). At 17 h, each animal was fed with the corresponding diets (STD or CAF) to obtain the values under *ad libitum* conditions. This procedure has been described by other authors previously [15]. Locomotor activity was also measured as previously described [8].

2.6. Fecal analysis

During the last week of the study, the frequency, weight and water content of the animals' feces were measured over a 24-h period. During the first 2 h, fresh feces were collected and quantified for consistency and water content measurements. The qualitative appearance of the fresh feces was evaluated using a three-point scale: normal, semi-solid, and diarrhea [16]. Water content was determined as the weight difference between fresh and dry stools obtained after a 24-h period at 70 °C, with the result being expressed as the percentage of fresh feces.

The same 24-h fecal samples were used for lipid absorption analysis. Fecal lipid content was analyzed using a gravimetry protocol described previously [17]. Lipid absorption was calculated from the total lipid content values in feces normalized for animals' lipid consumption with the results expressed as percentages.

2.7. Multi-omics analysis of serum, urine, cecum, and PBMC samples

Quantitative large-scale metabolomics analyses of serum and urine samples were conducted following the methodology described elsewhere with slight modifications [18]. ^{1}HNMR metabolic profiling analysis was performed following the procedure described by Palacios-Jordan et al. [19]. Detailed information about the metabolomic, transcriptomic and metagenomic methodologies employed in this study can be found in the Supplementary Material file.

2.8. Statistical analysis

All the data showed throughout the work are expressed as mean ± SEM. The Grubbs test and principal component analysis (PCA) were used to detect outliers, which were discarded for subsequent analyses. The homoscedasticity among groups was assessed using Levene's test, and when this condition was not accomplished, data were transformed to a base-10 logarithm to obtain similar variances before statistical testing. Variables within each data set (*i.e.*, biometrical/biochemical variables, NMR serum metabolomics, LC/MS serum metabolomics, LC/MS urine metabolomics, metagenomics) were compared among the experimental groups by using two-way analysis of variance (ANOVA), considering the diet (D: STD or CAF), the treatment (T: VH or F) and their interaction (D × T). For significant D × T interactions, Fisher's LSD post hoc and Student's t-tests were performed two-to-two among the different groups. The temporal evolution of some of the variables were analyzed by repeated-measures (RMs) ANOVA, with time as a within-subject factor and diet or treatment as a between-subject factor. All these statistical analyses were performed using the statistical software package SPSS Statistics 22 (SPSS Inc., Chicago, IL, USA). *P*-values <.05 were considered statistically significant.

For transcriptomic data analysis, sparse partial least squares discriminant analysis (sPLS-DA) using mixOmics package [20] was employed to select those genes with the greatest differences in expression between the two groups analyzed and with the greatest importance in component 1. The GeneSCF program (http://genescf.kandurilab.org/documentation.php) was then used to analyze which processes or metabolic pathways were most represented by the genes that showed the greatest differences in expression between groups, employing Fisher's exact test with various corrections for multiple comparisons. From the list of 200 selected genes, only those with a gene name or EntrezGeneID code were finally selected. The EntrezGeneID was used to search the *Rattus norvegicus* KEGG metabolic database (rno code in the KEGG database). To calculate the statistical parameters, 20,000 background genes were used.

Afterwards, multiple factor analysis (MFA) was performed for integrating all data sets with the aim of exploring the contribution of the different variables to the effects provoked by the treatment. MFA was performed by using only significant variables according to two-way ANOVA results for removing non-informative variables to sharpen the separation between the study groups. The MFA models were graphically displayed by plotting the projections of the variables (biometrical and biochemical; UHPLC-MS/MS metabolomics on serum and urine; NMR metabolomics on serum; metagenomics on family, genus and phylum levels; and transcriptomics) and samples (*i.e.*, group label) onto the bidimensional space defined by the first two dimensions. Finally, Pearson correlations were computed on significant variables (*P*-values <.05) to search for associations between metabolomics, metagenomics and biometrical/biochemical variables. Considering the differential impact of F treatment in STD- and CAF-fed animals, correlation analyses were conducted separately in each of the two diet groups. Correlations with *P*-values ≤.01 were considered significant and were graphically represented. All the data were normalized by means of log transformation and Pareto scaling prior to carrying out these statistical analyses using the R software packages FactoMineR and Factoextra (http://www.r-project.org).

3. Results and discussion

In this study, we evaluated the effect of a mixture of fibers with a high content of fermentable fibers (F), using a daily dose extrap-

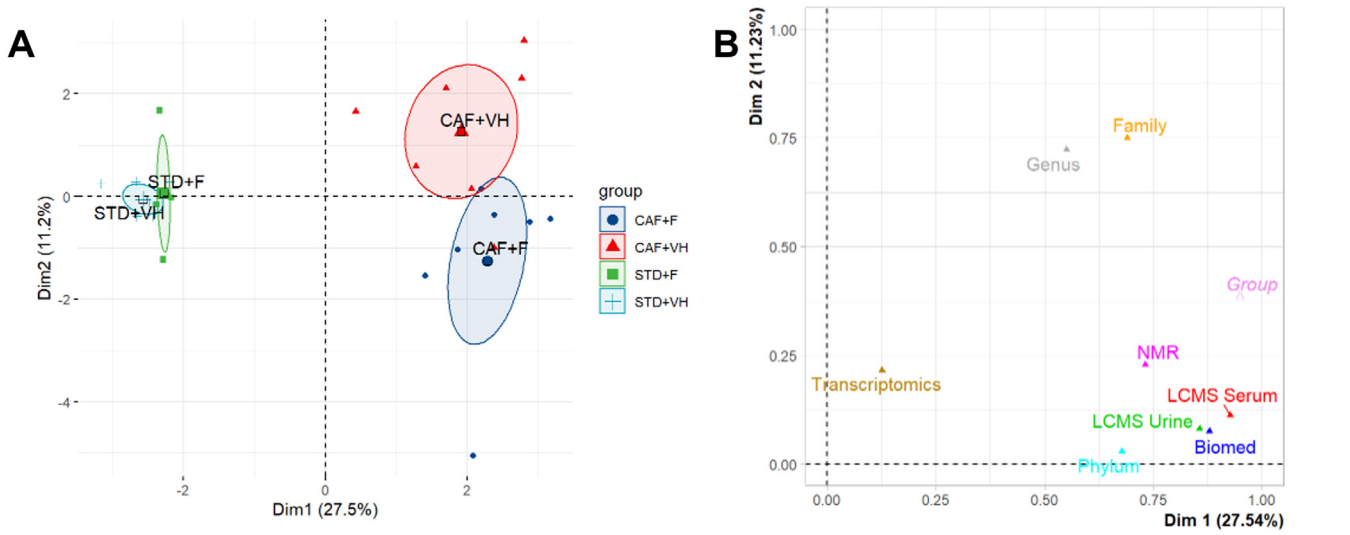

Fig. 1. Summary of multiple factor analysis (MFA) results. Scatter plots showing the projection of individual animals from the four study groups (*i.e.*, STD+VH, STD+F, CAF+VH, CAF+F) in the space defined by the first two dimensions, using the significant variables according to two-way ANOVA (A). Variable representation plots showing the projection of the different data sets (*i.e.*, biometrical/biochemical, NMR serum metabolomics, LC/MS serum metabolomics, LC/MS urine metabolomics, transcriptomics, metagenomics) in the space defined by the first two dimensions, using the significant variables according to two-way ANOVA (B).

olated to humans, on obesity and related disorders. Furthermore, we investigated the mechanisms and metabolic pathways potentially involved in the effects elicited by this intervention by means of the measurement of a wide number of biometric and biochemical parameters, the use of a multi-omics approach and their integration through multivariate analysis.

3.1. Multi-omics data integration

MFA modelling provided clear differentiation between CAF and STD groups along the first dimension, whereas F supplementation induced the separation of F-fed and VH-fed animals along the second dimension mainly in the CAF group (Fig. 1A). Overall, this is in line with the more pronounced response to the F treatment observed in CAF-fed animals, which could be understood as an adaptative mechanism to counteract the alteration of energy homeostasis produced by CAF consumption [8,17,21]. The variable representation plot (Fig. 1B) evidenced that biometrical and biochemical variables together with metabolomic data were the major drivers in the multivariate differentiation of the four study groups. Metagenomic variables also had a significant impact on the two first MFA dimensions, whereas the contribution of transcriptomic data was minor. Afterwards, Pearson's correlation analyses were conducted to investigate the association among the most significant variables according to two-way ANOVA and MFA results (Figs. 2 and 3). Altogether, this integrative multi-omics analysis enabled us to comprehensively elucidate the molecular mechanisms and biological pathways influenced by the F intervention, as detailed in the following sections.

3.2. F supplementation promotes a healthier body composition and metabolic profile

The results showed a clear reduction of body weight as a result of F treatment, with decreases of 11% and 6% in body weight gain in the STD+F and CAF+F groups, respectively (Table 1). In addition, a reduction in relative and absolute weights of EWAT depot and in absolute weight of MWAT depot was observed (Table 1), without affecting the energy intake of the animals (Supplementary Fig. 1). Furthermore, although no significant differences were observed regarding the percentage of fat mass, lean mass, lean mass:fat mass ratio or body water content during the intervention in either dietary group (Fig. 4A, 4C–E), F treatment significantly reduced the fat mass of the animals at the end of the study, with decreases of 6% and 16% in the STD+F and CAF+F groups, respectively (Fig 4B). In fact, F treatment significantly decreased the adiposity index and showed a clear tendency (two-way ANOVA, P=.05 for T) to reduce the visceral adipose index of the animals (Table 1). These results are relevant since excessive visceral adipose tissue accumulation has been shown to be closely related to the appearance of cardiovascular diseases, type 2 diabetes and increased mortality risk [22]. Interestingly, the body weight gain of the animals was positively correlated with MWAT depot, adiposity index and body fat mass (Fig 2), reinforcing the hypothesis that F treatment reduces body weight through a decrease of body fat in animals. This F-mediated enhancement of the body composition profile, without a significant reduction in energy intake, has been previously described by different authors in both animal and human studies [7,23]. Moreover, our results concur with several studies suggesting that fermentable fiber consumption produces a change in body fat distribution towards a healthier profile [7].

Leptin and adiponectin are considered plasma biomarkers of adipose tissue functionality, which are produced at levels proportional to the body fat content [21]. In this sense, agreeing with the observed F effect on fat reduction in CAF-fed animals, F treatment produced a global tendency to decrease the circulating levels of leptin (two-way ANOVA, P=.08 for T) and a significant decrease of adiponectin serum levels in CAF-fed animals (Student's t-test, P<.05) compared to animals treated with vehicle (Table 1). Contradictory results can be found in the literature in this regard, since various authors have reported that soluble fiber consumption is associated with an increase in adiponectin levels [24], whereas other studies did not detect any effect of fermentable fiber on this biomarker [25]. It is important to highlight that serum adiponectin levels relative to the adipose tissue mass did not differ as a result of the intervention (Table 1), thereby indicating an effective production of adiponectin by adipose tissue [26].

In transcriptomics analysis, significant differences were observed in several metabolic pathways in CAF fed animals, but not

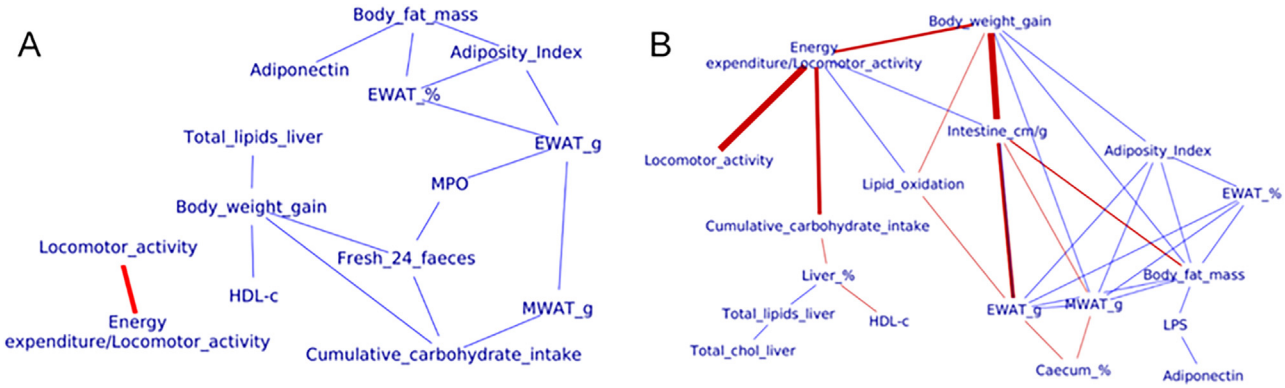

Fig. 2. Network representation of the associations between biometrical/biochemical parameters as a result of F treatment in animals fed the STD diet (A) and the CAF diet (B). Pearson correlations were computed on significant variables and significant correlations (*P*-value ≤.01) were represented. Positive correlations are shown in blue and negative correlations in red. The thickness of the line refers to the value of the correlation, the correlation being greater the thicker the line.

Table 1
Biometric and serum concentration parameters in rats fed a standard diet (STD) or cafeteria diet (CAF) and treated with vehicle (VH) or fiber (F)

	STD+VH	STD+F	CAF+VH	CAF+F	ANOVA
Initial body weight (g)	97.7 ± 2.30	99.0 ± 1.70	95.3 ± 1.70	96.5 ± 1.80	-
Final body weight (g)	374 ± 11	344 ± 6	447 ± 17	426 ± 14	D
Δ Body weight (g)	277 ± 11	245 ± 5	352 ± 16	330 ± 13	D, T
RWAT (g)	8.02 ± 0.51	7.08 ± 0.52	19.4 ± 1.30	17.1 ± 1.30	D
RWAT (%)	2.93 ± 0.21	2.80 ± 0.19	5.48 ± 0.19	5.12 ± 0.22	D
IWAT (g)	4.35 ± 0.32	3.91 ± 0.17	10.4 ± 1.10	8.2 ± 0.60	D
IWAT (%)	1.57 ± 0.09	1.55 ± 0.09	2.90 ± 0.17	2.50 ± 0.15	D
MWAT (g)	5.24 ± 0.21	4.43 ± 0.20	10.7 ± 0.80	9.3 ± 0.80	D, T
MWAT (%)	1.92 ± 0.10	1.76 ± 0.07	3.02 ± 0.13	2.80 ± 0.14	D
EWAT (g)	7.57 ± 0.41	5.75 ± 0.37	17.8 ± 1.40	14.8 ± 1.40	D, T
EWAT (%)	2.78 ± 0.18	2.29 ± 0.16	5.00 ± 0.26	4.41 ± 0.30	D, T
Adiposity index	9.19 ± 0.49	8.54 ± 0.40	16.4 ± 0.60	14.8 ± 0.70	D, T
Visceral adiposity index	7.63 ± 0.46	6.85 ± 0.35	13.5 ± 0.50	12.3 ± 0.60	D
Leptin (ng/mL)	9.23 ± 0.94	8.70 ± 0.91	47.21 ± 5.76	31.98 ± 2.98	D
Adiponectin (μg/mL)	21.95 ± 1.56	22.49 ± 2.66	34.96 ± 2.59	24.55 ± 1.36	D, T, DxT
Leptin:Adiponectin Ratio	0.40 ± 0.06	0.36 ± 0.04	1.36 ± 0.15	1.34 ± 0.14	D
Adiponectin:Fat mass Ratio	0.62 ± 0.99	0.73 ± 0,20	0.35 ± 0.09	0.31 ± 0.09	D

Wistar rats supplemented with fiber (F) or vehicle (VH) and fed a cafeteria (CAF) diet or standard (STD) diet for 8 weeks. EWAT, epididymal white adipose tissue; MWAT, mesenteric white adipose tissue; IWAT, inguinal white adipose tissue; and RWAT, retroperitoneal white adipose tissue is expressed as absolute weight (g) and relative weight (%), calculated according to the formula (100*tissue weight/body weight). The adiposity index and visceral adipose index were computed as the sum of EWAT, MWAT, IWAT and RWAT depot weights (in grams) or the sum of RWAT, IWAT and EWAT depot weights (in grams), respectively, and expressed as a percentage of body weight. Data are given as mean ± SEM. D, diet effect; T, fiber treatment effect; DxT, diet-treatment interaction (Two-way ANOVA test, *P*<.05).

when comparing STD+VH and STD+F groups (*data not shown*). In line with the results discussed above on the beneficial effect of F on body weight and body fat distribution, F treatment produced a significant change in the thermogenic pathway by decreasing the expression levels of several related genes in PBMCs, such as *NADH: ubiquinone oxidoreductase subunit C2, protein kinase cAMP-activated catalytic subunit beta and ATP synthase* (Table S1). In this regard, previous studies with animals have described that the consumption of high-fat diets, including the CAF diet, increases the thermogenic capacity in an attempt to dissipate excessive energy intake and maintain body weight energy [28].

3.3. The administration of F decreases locomotor activity and enhances energy expenditure and lipid oxidation in CAF-fed rats

F treatment produced a significant reduction in global activity (Fig. 5A), which could suggest a treatment effect on anxiety and stress, as reported in other studies [27]. No significant changes were observed in energy expenditure analysis (Fig. 5B). However, when corrected by animal activity, a significant energy expenditure enhancement was found in CAF-fed animals treated with F (Student's t-test, *P*<.05) (Fig. 5C). In addition, an increase in fat oxidation in the feeding period was observed in the CAF+F group (Student's t-test, *P*<.05) (Fig. 5D), but no significant effects were observed on carbohydrate oxidation (Fig. 5E) either in the CAF-fed or STD-fed groups. These results suggest that the increased energy expenditure seems to be due to enhanced lipid oxidation, a finding that was corroborated in correlation analysis, since lipid oxidation was positively correlated with energy expenditure and negatively correlated with the body weight of the animals fed with the CAF diet (Fig. 2B). Furthermore, in CAF-fed animals, carbohydrate intake negatively correlated with energy expenditure corrected by locomotor activity (Fig. 2B). In these animals, F treatment showed a tendency to decrease the preference for carbohydrate consumption (Student's t-test, *P*=.090) (Fig. 5E), which could be explained by the noticed less intake of sweetened milk (289±12 mL day^{-1}

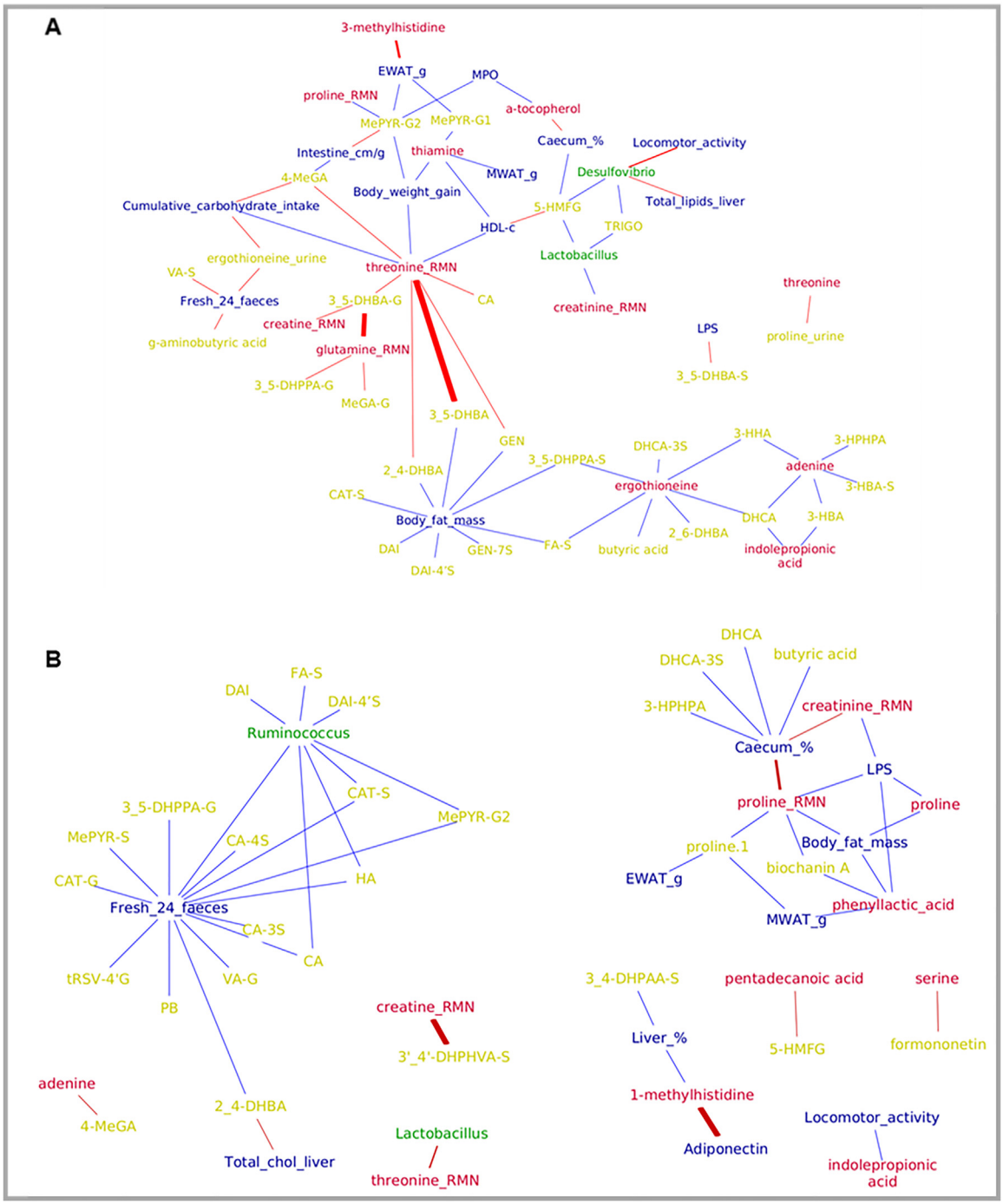

Fig. 3. Network representation of the associations between metabolomic, metagenomic and biometrical/biochemical variables as a result of F treatment in animals fed the STD diet (A) and the CAF diet (B). Pearson correlations were computed on significant variables and significant correlations (P-value $\leq$.01) were represented. Positive correlations are shown in blue and negative correlations in red. The thickness of the line refers to the value of the correlation, the correlation being greater the thicker the line. Label colors differentiate the different sets of measures: blue for biometrical/biochemical parameters; red for serum metabolomics; yellow for urine metabolomics; green for metagenomics.

in the CAF+VH group *vs.* 242±13 mL day^{-1} in the CAF+F group, Student's t-test, P=.016), the most consumed food in the CAF diet-fed animals. According to these results [28], F addition significantly reduced the circulating levels of pentadecanoic acid in CAF fed animals (Table 2), a marker of the intake of dairy products [28]. These results suggest that the reduction in carbohydrate intake could mediate, along with the increase in lipid oxidation, the beneficial effects of F on body weight and adiposity reduction in CAF diet-fed animals treated with F.

3.4. F supplementation improves cholesterol metabolism and HbA1c blood levels

No significant effects of F treatment were observed in CAF-fed animals on total cholesterol, LDL cholesterol, triglyceride, or NEFAs blood levels, nor on lipid absorption. However, in STD-fed animals, F treatment produced a tendency towards a reduction in LDL cholesterol serum levels (Student's t-test, P<.1) and significantly decreased total cholesterol levels in the liver (Student's t-

Table 2
Metabolomic alterations induced in serum and urine by the dietary intervention with fibres in rats fed standard diet (STD) or cafeteria diet (CAF). Metabolite concentrations are expressed as mean ± standard deviation (μg L^{-1} for UHPLC-MS/MS measurements, nmol L^{-1} for ^{1}H-NMR measurements)

Metabolite	STD+VH	STD+F	CAF+VH	CAF+F	ANOVA
UHPLC-MS/MS serum					
amino acids and derivatives					
serine	485.0 ± 187.6	627.0 ± 96.2	941.5 ± 442.4	638.7 ± 116.2	T, DxT
threonine	2932.5 ± 791.2	3655.5 ± 244.8	5002.6 ± 1371.0	3970.9 ± 1276.0	T, DxT
proline	11064.0 ± 2556.5	11657.2 ± 1204.9	14848.2 ± 3443.5	10278.9 ± 2036.1	T, DxT
1-methylhistidine	311.8 ± 64.3	288.4 ± 90.8	356.4 ± 89.0	535.8 ± 168.4	D, T, DxT
3-methylhistidine	1721.1 ± 516.8	2076.3 ± 372.7	1909.6 ± 748.4	2744.9 ± 940.6	T
ergothioneine	587.1 ± 150.1	644.5 ± 121.9	261.9 ± 51.6	202.6 ± 20.8	D, T, DxT
microbiota metabolites - aromatic amino acids					
phenyllactic acid	24.3 ± 14.3	28.2 ± 14.1	53.2 ± 45.3	23.8 ± 13.7	T, DxT
phenylacetylglycine	191.9 ± 154.1	217.0 ± 109.6	140.5 ± 78.5	74.3 ± 31.3	D, T, DxT
phenylacetylglutamine	5.9 ± 6.2	8.7 ± 6.2	14.8 ± 19.9	3.5 ± 2.4	T, DxT
indolepropionic acid	785.3 ± 228.4	668.0 ± 229.2	924.1 ± 410.6	618.7 ± 575.0	T
vitamins					
thiamine	1387.6 ± 486.5	925.0 ± 280.3	762.8 ± 134.1	935.1 ± 285.4	DxT
riboflavin	81.3 ± 23.3	68.8 ± 13.2	62.3 ± 8.7	52.2 ± 6.6	D, T
α-tocopherol	9620.3 ± 2186.9	6559.9 ± 2583.2	5907.9 ± 1552.7	6507.9 ± 910.7	DxT
free fatty acids					
pentadecanoic acid	ND	ND	8232.5 ± 3510.4	3528.4 ± 2959.3	D, T, DxT
amines					
adenine	16.1 ± 4.8	15.0 ± 2.5	16.4 ± 3.9	12.3 ± 2.1	T
^{1}H-NMR serum					
Creatine	0.1 ± 0.004	0.13 ± 0.01	0.14 ± 0.01	0.16 ± 0.01	D, T
Creatinine	9.25 ± 0.45	10.4 ± 0.30	10.9 ± 0.72	9.63 ± 0.60	DxT
Phenylalanine	44.7 ± 1.38	45.4 ± 1.35	48.8 ± 1.35	42.0 ± 0.799	D, T, DxT
Proline	0.094 ± 0.01	0.087 ± 0.01	0.112 ± 0.01	0.088 ± 0.004	T
Glutamine	0.28 ± 0.01	0.31 ± 0.01	0.32 ± 0.01	0.27 ± 0.01	DxT
Threonine	0.12 ± 0.01	0.13 ± 0.004	0.18 ± 0.01	0.15 ± 0.003	D, T, DxT
UHPLC-MS/MS urine					
amino acids and derivatives					
proline	977.4 ± 304.9	791.2 ± 267.5	1212.0 ± 689.1	808.4 ± 311.8	T
ergothioneine	514.7 ± 94.9	543.0 ± 66.3	371.2 ± 46.7	426.9 ± 57.6	D, T
microbiota metabolites - aromatic amino acids					
phenylacetylglycine	68959.4 ± 15461.7	52043.1 ± 11411.9	40407.6 ± 12765.8	31564.8 ± 16129.9	D, T
microbiota metabolites - phenolic acids					
3-hydroxybenzoic acid (3-HBA)	54.1 ± 48.9	47.0 ± 45.8	35.8 ± 13.3	75.8 ± 40.9	D, T, DxT
3-hydroxybenzoic acid sulfate (3-HBA-S)	8.8 ± 7.2	9.5 ± 9.8	5.8 ± 2.6	23.0 ± 27.1	D, T, DxT
2,4-dihydroxibenzoic acid (2,4-DHBA)	23.2 ± 8.8	18.0 ± 10.0	4.8 ± 2.1	6.9 ± 2.6	D, T, DxT
2,6-dihydroxibenzoic acid (2,6-DHBA)	277.9 ± 96.4	226.9 ± 68.9	78.2 ± 54.5	105.3 ± 43.6	D, T, DxT
3,4-dihydroxibenzoic acid (3,4-DHBA)	557.7 ± 1167.1	345.9 ± 339.5	349.8 ± 205.0	719.9 ± 322.0	D, T
3,5-dihydroxibenzoic acid (3,5-DHBA)	581.7 ± 512.2	321.4 ± 171.4	116.2 ± 52.9	251.0 ± 177.8	T, DxT
3,5-dihydroxibenzoic acid glucuronide (3,5-DHBA-G)	19.6 ± 11.3	11.1 ± 6.0	5.3 ± 3.8	20.8 ± 23.7	T, DxT
3,5-dihydroxibenzoic acid sulfate (3,5-DHBA-S)	44.8 ± 13.8	34.4 ± 15.8	18.9 ± 14.0	68.1 ± 99.5	T, DxT
hippuric acid (HA)	206091.4 ± 121497.1	204201.2 ± 57038.3	74803.0 ± 36328.0	123096.7 ± 37038.3	D, T
3-hydroxyhippuric acid (3-HHA)	284.2 ± 327.9	255.6 ± 278.3	108.8 ± 50.4	226.1 ± 106.1	T
vanillic acid glucuronide (VA-G)	96.4 ± 34.2	80.5 ± 30.8	77.2 ± 36.1	113.9 ± 51.3	D, T, DxT
vanillic acid sulfate (VA-S)	270.8 ± 63.3	290.2 ± 95.7	394.2 ± 95.1	765.1 ± 712.4	D, T, DxT
4-methylgallic acid (4-MeGA)	6.9 ± 4.4	8.9 ± 4.1	9.9 ± 4.7	21.2 ± 10.2	D, T
3-methylgallic acid (3-MeGA)	7.9 ± 5.9	5.5 ± 2.4	5.7 ± 3.2	8.2 ± 3.5	D, T, DxT
methylgallic acid glucuronide (MeGA-G)	5.1 ± 1.3	4.5 ± 1.8	3.2 ± 2.7	5.2 ± 2.9	T, DxT
3,4-dihydroxyphenylacetic acid sulfate (3,4-DHPAA-S)	60641.8 ± 22766.5	58082.5 ± 12291.9	38190.4 ± 6702.8	50329.4 ± 10990.4	T, DxT
p-coumaric acid glucuronide (pCOU-G)	21.0 ± 15.0	21.0 ± 27.0	2.6 ± 5.2	7.5 ± 7.0	DxT
m-coumaric acid sulfate (mCOU-S)	13215.6 ± 9417.5	13538.9 ± 11199.4	2705.3 ± 1444.6	5234.4 ± 3643.7	D, T
caffeic acid (CA)	64.1 ± 31.3	59.6 ± 34.4	27.7 ± 19.0	60.8 ± 22.6	T, DxT
caffeic acid 3-sulfate (CA-3S)	20.8 ± 20.2	19.2 ± 21.5	5.3 ± 4.9	14.0 ± 14.5	T, DxT
caffeic acid 4-sulfate (CA-4S)	32.6 ± 7.9	35.2 ± 12.9	18.5 ± 10.7	46.9 ± 49.2	T, DxT
ferulic acid glucuronide (FA-G)	875.8 ± 825.1	1103.7 ± 1515.7	63.6 ± 172.9	104.1 ± 131.6	D, T, DxT
ferulic acid sulfate (FA-S)	7252.5 ± 4845.7	4961.5 2607.7	2058.9 ± 1412.3	2665.6 ± 1221.0	D, T, DxT
3,5-dihydroxyphenylpropionic acid glucuronide (3,5-DHPPA-G)	3393.5 ± 614.0	3028.7 ± 1162.4	1982.7 ± 1709.5	3237.2 ± 1851.9	T, DxT
3,5-dihydroxyphenylpropionic acid sulfate (3,5-DHPPA-S)	19803.8 ± 12391.2	17193.2 ± 6440.8	3424.0 ± 2187.4	5088.9 ± 1932.2	D, T, DxT
dihydrocaffeic acid (DHCA)	105.5 ± 82.0	95.3 ± 55.1	73.0 ± 23.8	132.1 ± 67.3	T, DxT
dihydrocaffeic acid 3-sulfate (DHCA-3S)	569.5 ± 242.5	519.0 ± 219.0	173.4 ± 68.2	319.1 ± 200.6	D, T, DxT

(*continued on next page*)

Table 2 (*continued*)

Metabolite	STD+VH	STD+F	CAF+VH	CAF+F	ANOVA
dihydroferulic acid sulfate (DHFA-S)	1660.0 ± 1739.6	833.7 ± 307.2	531.8 ± 429.1	760.3 ± 475.9	DxT
3-(hydroxyphenyl)-hydroxypropionic acid (3-HPHPA)	13.8 ± 19.6	11.5 ± 17.3	6.2 ± 3.9	18.4 ± 12.9	D, T, DxT
methylpyrogallol glucuronide (isomer 1) (MePYR-G1)	27.0 ± 5.9	18.8 ± 7.8	30.8 ± 22.0	45.2 ± 25.0	D, T, DxT
methylpyrogallol glucuronide (isomer 2) (MePYR-G2)	193.1 ± 75.0	109.0 ± 63.4	124.9 ± 118.9	281.2 ± 301.0	DxT
methylpyrogallol sulfate (isomer 2) (MePYR-S2)	79.4 ± 83.4	42.5 ± 39.9	53.9 ± 29.1	145.2 ± 154.1	D, T, DxT
catechol glucuronide (CAT-G)	283.6 ± 360.3	372.5 ± 655.6	336.9 ± 333.1	1015.0 ± 1028.6	D, T, DxT
catechol sulfate (CAT-S)	775228.5 ± 292572.3	702821.1 ± 147289.5	323083.2 ± 112463.6	438712.1 ± 137717.0	D, T, DxT
short chain fatty acids					
butyric acid	3980.2 ± 1442.7	4009.1 ± 1166.1	1842.5 ± 805.6	2590.6 ± 1013.5	D, T
γ-aminobutyric acid	268.7 ± 75.9	286.3 ± 93.3	205.7 ± 89.6	288.5 ± 74.5	T
food-related metabolites – hydroxyphenyl-valerolactones					
4-hydroxy-5-(3′,4′-dihydroxyphenyl)-valeric acid sulfate (3,4-DHPVA-S)	3.6 ± 6.4	5.3 ± 7.5	14.1 ± 9.5	6.4 ± 9.4	T, DxT
5-(3′,4′-dihydroxyphenyl)-γ-valerolactone 3′-glucuronide (3,4-DHPV-3G)	ND	0.5 ± 0.9	1.8 ± 0.8	1.4 ± 1.0	D, DxT
food-related metabolites - isoflavones					
daidzein (DAI)	1058.6 ± 505.1	706.9 ± 229.8	271.1 ± 189.8	421.5 ± 181.6	D, T, DxT
daidzein 4′-sulfate (DAI-4'S)	2504.6 ± 1692.9	1451.2 ± 648.5	737.5 ± 564.2	1200.4 ± 548.0	T, DxT
genistein (GEN)	830.5 ± 508.1	492.5 ± 210.8	256.3 ± 204.1	345.3 ± 175.9	T, DxT
genistein 7-sulfate (GEN-7S)	97.4 ± 137.6	29.4 ± 35.6	29.7 ± 34.6	38.6 ± 32.7	DxT
biochanin A (BioA)	16.7 ± 15.2	6.0 ± 3.7	5.5 ± 6.0	6.9 ± 4.2	DxT
formononetin (FOR)	3.3 ± 1.4	2.5 ± 1.4	1.5 ± 1.0	2.3 ± 0.8	T, DxT
food-related metabolites - flavones					
luteolin (LUT)	1.4 ± 0.7	0.7 ± 0.5	0.6 ± 0.4	1.2 ± 1.1	T, DxT
food-related metabolites - stilbenoids					
trans-resveratrol 4′-glucuronide (tRSV-4'G)	16.4 ± 9.8	7.2 ± 4.7	3.0 ± 3.0	4.9 ± 2.9	DxT
food-related metabolites - betaines					
proline betaine	3579.8 ± 857.1	3633.1 ± 938.7	812.7 ± 481.7	1159.3 ± 468.7	D, T
trigonelline	32874.2 ± 9812.2	39288.0 ± 18354.8	551.2 ± 1743.2	2803.9 ± 3592.7	D, T, DxT
food-related metabolites - others					
5-(hydroxymethyl-2-furoyl)glycine (5-HMFG)	136.7 ± 35.8	1445.4 ± 557.0	155.5 ± 64.2	1715.5 ± 716.1	T

test, $P<.05$) (Table 3). The treatment with F did not produce any effect either on total liver lipids or on triglyceride levels either in STD-fed groups or in CAF-fed groups (Table 2,3). These results are in line with other studies demonstrating that fiber consumption produces a reduction in LDL cholesterol without affecting triglyceride levels [7]. This effect could be associated with the fermentation of soluble fiber by the intestinal microbiota and the production of short-chain fatty acids (SCFAs), which inhibit liver cholesterol synthesis [29], although in our study no correlations were observed between serum or liver LDL levels and SCFAs in STD-fed animals (Fig. 2A). It should be also mentioned that the CAF+F group showed a significant increase in liver relative weight compared to the CAF+VH group (Student's t-test, $P<.05$), while no effects were observed between STD-fed groups. This could be due to the observed reduction of body weight in F-treated groups or to factors other than the fat content in this organ, such as increased blood supply. In contrast, our results were not consistent with different studies describing that fiber consumption produces either no effect or an increase in plasma HDL cholesterol levels [7]. The reduction of HDL cholesterol by F treatment would be a non-beneficial effect (Table 3), since this cholesterol fraction is related to lower risk of cardiovascular disease development by decreasing the risk of atherosclerosis [30]. However, it should be borne in mind that, in order to confirm a harmful risk of F treatment, more direct markers should be analyzed, such as endothelial function. Furthermore, other markers associated with an increase in cardiovascular risk, such as apolipoprotein B-100 serum levels [30], LDLox and TBARS, were not altered by F treatment (Table 3).

Regarding glucose metabolism, although no effects were observed on glucose and insulin levels, HOMA-IR and R-QUICKI indexes (Table 3) or OGTT (*data not shown*) by F treatment, a significant reduction of HbA1c, which is considered a plasmatic indicator of long-term glucose levels, was found in both STD+F and CAF+F groups (Table 3). These results are consistent with some studies in which soluble fiber reduced HbA1c levels in rat models of obesity and diabetes [31], indicating a beneficial effect of fiber on glucose control.

3.5. F supplementation improves intestinal health and reduces endotoxaemia

CAF+F animals presented higher stool fresh weight (Student's t-test, $P<.05$), which indicates beneficial effects of F on constipation (*data not shown*). These results concur with the reported beneficial effect of dietary fiber and dried plums on intestinal function mediated by the increase of bowel movement frequency and stool weight [32,33]. Furthermore, F treatment produced an increase in cecal weight (%) in both dietary groups (Table 4). This increase has been previously allocated to a rise in epithelial cell proliferation as a result of the trophic effect of SCFAs produced during fiber fermentation in the cecum by microbiota [34]. The length of the intestines is considered a good indicator of intestinal inflammation, as the shorter their length, the greater the intestinal inflammation [35–37]. In this respect, F treatment also increased the small intestine length:weight ratio in both dietary groups (Table 4), suggesting a decrease in the inflammatory state of this tissue

Table 3
Serum, liver, and fecal parameters related to lipid and glucose metabolism, systemic inflammation and oxidative system in rats fed standard diet (STD) or cafeteria diet (CAF) and treated with vehicle (VH) or fiber (F)

	STD+VH	STD+F	CAF+VH	CAF+F	ANOVA
Plasmatic parameters					
Total Chol (mg/dL)	108 ± 4.72	100 ± 3.53	107 ± 3.97	111 ± 5.13	-
LDL-c (mg/dL)	45.5 ± 2.05	40.7 ± 1.55	51.3 ± 1.86	58.8 ± 4.14	D, DxT
HDL-c (mg/dL)	97.1 ± 3.23	86.7 ± 3.37	81.1 ± 4.03	66.5 ± 2.58	D, T
Triglycerides (mg/dL)	83.7 ± 9.11	72.8 ± 10.91	232 ± 17.62	268 ± 30.98	D
NEFAs (mmol/L)	0.45 ± 0.06	0.38 ± 0.03	0.49 ± 0.04	0.45 ± 0.03	-
ApoB-100 (mg/mL)	2.57 ± 0.24	2.13 ± 0.24	2.66 ± 0.22	2.52 ± 0.27	-
LDLox (ng/mL)	20.1 ± 2.04	19.8 ± 1.80	26.8 ± 3.78	20.2 ± 0.78	-
TBARS (μmol/L)	13.0 ± 2.16	19.6 ± 3.24	24.3 ± 3.78	28.4 ± 2.22	D
Glucose (mg/dL)	111 ± 2.46	105 ± 3.07	123 ± 3.13	122 ± 3.50	-
Insulin (ng/mL)	1.02 ± 0.11	0.93 ± 0.07	1.81 ± 0.14	1.55 ± 0.17	D
HOMA-IR	6.35 ± 0.95	6.05 ± 0.53	13.8 ± 1.18	12.4 ± 1.66	D
R-QUIKI	0.34 ± 0.01	0.34 ± 0.01	0.29 ± 0.005	0.30 ± 0.01	D
HbA1c (μg/mL)	27.6 ± 1.30	21.6 ± 1.30	28.0 ± 1.10	24.6 ± 1.60	T
MCP-1 (ng/mL)	6.30 ± 0.35	6.92 ± 0.40	8.25 ± 0.36	8.18 ± 0.15	D
Hepatic parameters					
Liver (g)	11.4 ± 0.50	10.2 ± 0.30	14.6 ± 0.60	14.9 ± 0.40	D
Liver (%)	4.13 ± 0.08	4.02 ± 0.09	4.05 ± 0.06	4.39 ± 0.07	DxT
Total Lipid (mg)	382 ± 20	343 ± 18	673 ± 49	852 ± 92	D, DxT
Lipid (mg/g)	33.7 ± 1.7	33.6 ± 1.4	49.9 ± 4.6	58.5 ± 5.3	D
Total Chol (mg)	25.0 ± 1.5	20.8 ± 1.2	53.0 ± 7.5	70.6 ± 6.8	D, DxT
Chol (mg/g)	2.21 ± 0.14	2.06 ± 0.12	4.19 ± 0.41	4.83 ± 0.36	D
Total Triglycerides (mg)	62.8 ± 4.5	53.5 ± 4.2	163 ± 27	187 ± 19	D
Triglycerides (mg/g)	5.53 ± 0.41	5.29 ± 0.40	12.5 ± 1.4	12.8 ± 1.0	D
Fecal parameters					
Lipid content (mg/g feces)	18 ± 1.48	16.7 ± 0.46	26.8 ± 1.80	24.3 ± 2.87	D
Lipid content (mg)	70.8 ± 7.21	58.1 ± 3.65	35.7 ± 4.70	35.5 ± 4.62	D
Lipid absorption (g)	1.22 ± 0.05	1.13 ± 0.04	3.90 ± 0.18	4.33 ± 0.26	D

Wistar rats supplemented with fiber (F) or vehicle (VH) and fed a cafeteria (CAF) diet or standard (STD) diet for eight weeks. Chol; cholesterol, NEFAS; non-esterified fatty acid, ox-LDL; oxidized low-density lipoprotein, HbA1c; glycated hemoglobin, MCP-1; monocyte chemoattractant protein-1. Data are given as means ± SEM. D, diet effect; T, fiber treatment effect; DxT, diet-treatment interaction (Two-way ANOVA test, $P<.05$).

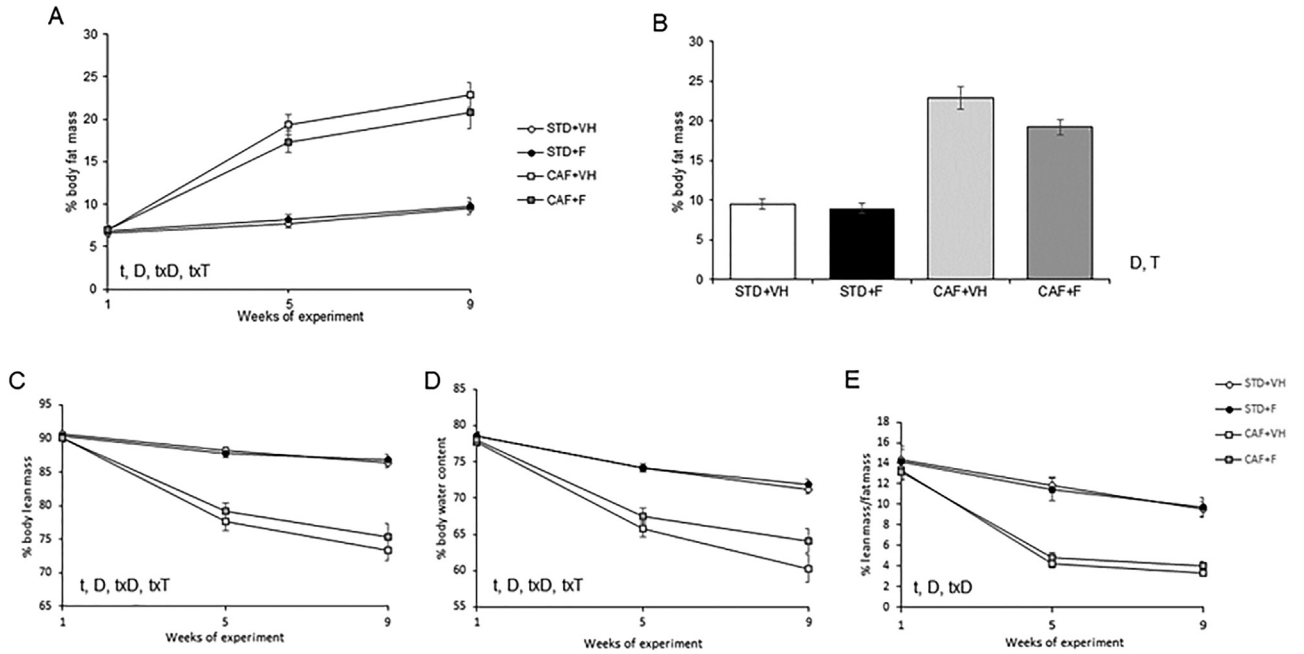

Fig. 4. Body composition parameters of rats fed a standard (STD) or cafeteria (CAF) diet and treated with vehicle (VH) or fiber (F). (A) Body fat mass evolution, (B) Body fat mass at the end of the study, (C) Body lean mass evolution, (D) Body water content evolution, (E) Ratio between body lean and fat mass evolution. The data are presented as the mean ± SEM. D, the effect of the diet; T, the effect of the fiber treatment; t, the effect of the time; txD, the interaction between time and diet; txT, the interaction between time and treatment (Two-way ANOVA and RMs ANOVA, $P<.05$).

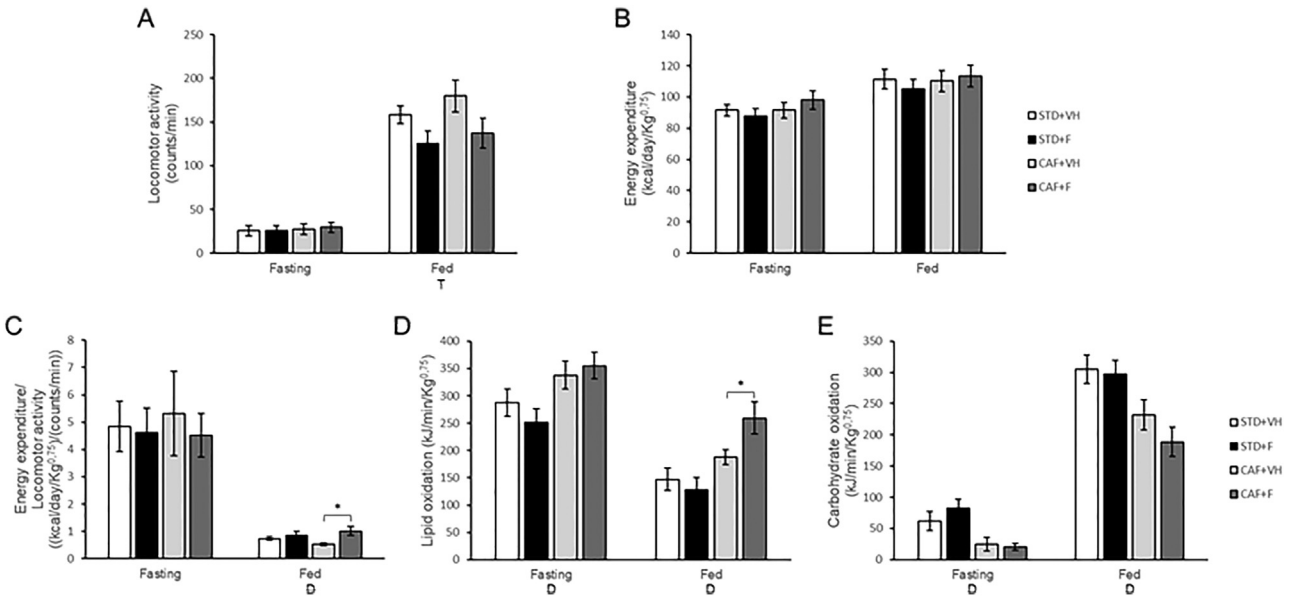

Fig. 5. Indirect calorimetry and locomotor activity of rats fed standard (STD) or cafeteria (CAF) diet and treated with vehicle (VH) or fiber (F). (A) Locomotor activity. (B) Energy expenditure. (C) Ratio between energy expenditure and locomotor activity. (D) Lipid oxidation. (E) Carbohydrate oxidation. The data are presented as the mean ± SEM. D, the effect of the diet; T, the effect of the fiber treatment; DxT, the interaction between diet and treatment (Two-way ANOVA, $P<.05$). *Significantly different between groups (Student's t-test, $P<.05$).

Table 4
Intestinal parameters and endotoxemia in rats fed standard diet (STD) or cafeteria diet (CAF) and treated with vehicle (VH) or fiber (F)

	STD+VH	STD+F	CAF+VH	CAF+F	ANOVA
Cecum (g)	5.31 ± 0.38	5.78 ± 0.28	3.44 ± 0.23	3.91 ± 0.17	D
Cecum (%)	1.82 ± 0.07	2.29 ± 0.11	0.99 ± 0.07	1.20 ± 0.06	D, T
Intestine (cm)	120 ± 1	118 ± 1	122 ± 2	121 ± 2	-
Intestine (cm/g)	15.5 ± 0.5	16.6 ± 0.6	13.3 ± 0.6	14.4 ± 0.4	D, T
Colon (cm)	21.4 ± 0.4	19.4 ± 0.6	18.3 ± 0.6	18.7 ± 0.4	D, DxT
Colon (cm/g)	14.6 ± 0.4	15.3 ± 0.6	16.0 ± 0.9	16.5 ± 0.6	-
MPO (U/mg tissue)	0.07 ± 0.01	0.04 ± 0.00	0.05 ± 0.01	0.04 ± 0.01	T
LPS (nM)	41.9 ± 5.61	53.2 ± 8.8	153.50 ± 27.1	62 ± 10.8	D, DxT

Wistar rats supplemented with fiber (F) or vehicle (VH) and fed a cafeteria (CAF) diet or standard (STD) diet for 8 weeks. MPO; myeloperoxidase, LPS; lipopolysaccharides. Cecum weight is expressed as absolute weight (g) and relative weight (%), calculated according to the formula (100*tissue weight/body weight) and expressed as percentage of body weight. Data are given as mean ± SEM. D, diet effect; T, fiber treatment effect; DxT, diet-treatment interaction (Two-way ANOVA test, $P<.05$).

[35–37]. In contrast, F treatment decreased colon length in the STD-fed group (Student's t-test, $P<.05$). Although this result could suggest increased colon inflammation as a result of F treatment, it should be borne in mind that this effect was only observed in animals fed the STD diet, where no external inducers of intestinal inflammation were observed, as indicated by MCP1 circulant levels (Table 3). In addition, F treatment produced a significant reduction of MPO activity in both STD and CAF groups (Table 4), suggesting that F treatment induces a decrease in inflammation at the colon level in both groups.

Furthermore, F treatment showed a clear reduction in LPS serum levels in CAF-fed animals (Student's t-test, P $P<.05$) (Table 4). These results are consistent with several *in vivo* [38] and human intervention studies [25], and reinforce that fermentable fiber might alter the composition and/or activity of intestinal microbiota, leading to a decrease in intestinal permeability and LPS plasma levels [38]. Interestingly, the body fat of animals was positively correlated with LPS levels and negatively correlated with cecum weight and intestine length (Fig. 2). The main consequence of intestinal barrier dysfunction is the entry of toxins, including LPS, causing metabolic endotoxemia [39,40]. Moreover, the results of numerous studies suggest that changes in the function of the intestinal barrier and intestinal inflammation are associated with, and could induce, metabolic alterations, including systemic inflammation, dysregulation of body weight or changes in glucose homeostasis [41]. Overall, our results suggest that F has beneficial effects on intestinal inflammation, permeability and health, and that the clear effect of F treatment on endotoxemia in CAF-fed animals could be associated with the beneficial effects observed on body weight, fat mass and glucose metabolism.

3.6. Involvement of metabolites derived from F supplementation in adiposity, lipid metabolism, and intestinal health: SCFAs, phenolic acids, and vitamins

Metabolomic profiles of urine and, to a lesser extent, serum samples showed alterations in metabolites that could be regarded as direct or indirect biomarkers of the F intervention (Table 2). Urinary levels of 5-(hydroxymethyl-2-furoyl)glycine were increased as a consequence of the F treatment in both study groups ($P<.001$), in line with previous works reporting that furfural-related metabolites are normally excreted after the consumption of fiber-rich

Table 5
Significantly changed microbiota between rats fed standard diet (STD) or cafeteria diet (CAF) and treated with vehicle (VH) or fibre (F)

Taxonomic level	STD+VH	STD+F	CAF+VH	CAF+F	ANOVA
Firmicutes	79.4 ± 2.62	78.3 ± 4.08	55.3 ± 4.57	51.5 ± 6.99	D
Lactobacillaceae	1.61 ± 0.26	4.45 ± 0.67	10.1 ± 1.19	15.6 ± 5.39	D, T
Lactobacillus	1.61 ± 0.26	4.45 ± 0.66	10.1 ± 1.91	15.6 ± 5.38	D, T
Christensenellaceae Ruminococcaceae	0.010 ± 0.002	0.018 ± 0.004	0.045 ± 0.012	0.015 ± 0.005	D, DxT
Ruminococcus	1.16 ± 0.10	1.38 ± 0.19	0.58 ± 0.21	1.48 ± 0.30	T
Bacteroidetes	18.9 ± 2.62	19.1 ± 3.71	35 ± 4.18	38.8 ± 5.23	D
Porphyromonadaceae	0.176 ± 0.032	0.347 ± 0.101	1.66 ± 0.38	4.00 ± 1.04	D, T
Parabacteroides	0.1175 ± 0.032	0.346 ± 0.101	1.66 ± 0.38	4.00 ± 1.04	D, T
Paraprevotellaceae	$2.06{\cdot}10^{-3}$ ± $8.23{\cdot}10^{-4}$	0.028 ± 0.016	0.059 ± 0.030	0.007 ± 0.003	DxT
Proteobacteria	0.20 ± 0.05	0.39 ± 0.14	2.56 ± 0.88	2.42 ± 0.46	D
Desulfovibrionaceae Desulfovibrio	$3.30{\cdot}10^{-4}$ ± $1.78{\cdot}10^{-4}$	0.002 ± $9.63{\cdot}10^{-4}$	0.004 ± 0.001	0.001 ± $3.58{\cdot}10^{-4}$	DxT

Wistar rats supplemented with fibre (F) or vehicle (VH) and fed a cafeteria (CAF) diet or standard (STD) diet for 8 weeks. **Phylum;** Family; Genus.

foods [42]. We also observed an increase of SCFAs (butyrate, P=.049; gamma-aminobutyrate, P=.015), which are produced by bacterial fermentation of non-digestible fibers [43]. Furthermore, urinary levels of multiple phenolic acids and flavonoids were significantly increased in the CAF group as a direct effect of the F treatment (P<.05), but the opposite trend (not reaching statistical significance, P<.1) was observed in STD-fed animals. Similarly, F intake also provoked the reduction of various serum vitamins in the STD group (*e.g.*, thiamine, P=.016; α-tocopherol, P=.009), whereas their concentrations were slightly increased in the CAF-fed group. These apparently contradictory results could be attributed to the fact that the plum employed in this study is expected to increase the supply of these plant-related bioactives in animals fed with the CAF diet, which is enriched in unhealthy foods. On the other hand, F supplementation could reduce the absorption of polyphenols and vitamins that are naturally present in the cereal-containing chow that was employed in the STD group, thus decreasing their circulating levels, in line with previous studies [44].

As a result of this differential metabolomics effect of F between STD-fed and CAF-fed animals, correlation analyses showed opposite directions of association according to the diet group (Figure 3). SCFAs and phenolic acids were positively correlated with intestinal health-related parameters in CAF-fed animals (*e.g.*, cecum relative weight with butyric acid, DHCA, DHCA-3S and 3-HPHPA, and 24-h feces weight with CA derivatives and other phenolics), whereas the opposite trend was observed in the STD group (*e.g.*, 24-h feces weight with aminobutyric acid and VA-S). Similarly, these metabolite classes were significantly correlated with several adiposity variables (*e.g.*, positive correlation with fat mass, weight gain and EWAT in STD animals, negative correlation with total liver cholesterol in CAF animals). Therefore, these results could suggest that the beneficial effects of F treatment on obesity, liver lipid metabolism and intestinal health are mediated, at least in part, by SCFAs and phenolic acids.

In turn, we observed an increased abundance of the *Ruminococcus* genus in response to the dietary intervention with F (Table 5), which was positively correlated with urinary levels of phenolic acids and with fresh stool weight in CAF-fed animals (Fig. 3). In this respect, various authors have previously described an increase of these saccharolytic species when adding fermentable fiber or carbohydrates to the diet [45], bacteria that are responsible for the first steps of the microbiota-derived degradation and fermentation of carbohydrates. Notably, *Ruminococcus* species belong to the *Clostridium* cluster IV, which are also essential for the bioavailability of most polyphenols by driving C-ring cleavage reactions that lead to the production of simpler phenolic acids [46].

Within this tangled interplay between phenolic compounds and the gut microbiota, F treatment also altered the expression of genes involved in tight junction functioning, downregulating the *protein kinase cAMP-activated catalytic subunit beta, radixin* and *protein kinase C, iota* (Table S1). This is in line with previous studies reporting that dietary phenolics can regulate the synthesis, expression, and redistribution of tight junction proteins [47]. Tight junctions are the principal determinants of epithelial paracellular permeability and help to regulate epithelial barrier properties [46]. They are organized by specific interactions between a wide spectrum of proteins, including the integral role of the claudin family of transmembrane proteins [47]. The results obtained in our study concur with previous studies reporting the expression of genes related to tight junction processes in PBMCs under several conditions [48,49]. In particular, it should be noted that the interaction of LPS with human small intestinal lamina propria fibroblasts has been shown to favor PBMC adhesion through the production of adhesion molecules [50]. Furthermore, obesity induced by the intake of a high-fat diet triggers gut hyperpermeability through tissue-specific claudin switching in the gut epithelium of obese rather than lean subjects [49], in line with the upregulated expression levels of claudin observed in our study.

3.7. F modulates proteolysis and circulating amino acid levels

The intervention with F reduced serum and urinary amino acids towards similar levels to those detected in STD-fed rats (Table 2, P<.05). These amino acids were positively correlated with various adiposity-related parameters (*e.g.*, MWAT, EWAT and body fat mass with proline) in CAF animals (Fig. 3), thus evidencing the great importance of their proper homeostasis in regulating the metabolic complications promoted by this obesogenic diet. These findings concur with previous studies reporting that F treatment may significantly influence the metabolism of amino acids through different mechanisms, including enhanced hepatic gluconeogenesis [48], decreased proteolysis [49] and increased efficiency of amino acid utilization to maintain protein synthesis [45].

Moreover, serum amino acids (*i.e.*, threonine) were negatively correlated with the cecal abundance of the *Lactobacillus* genus (Fig. 3). F feeding induced an increase in *Lactobacillus* counts, in line with previous studies indicating that dietary fibers provoke important alterations in the gut microbiota composition to-

wards increased saccharolytic species and decreased detrimental proteolytic bacteria [49]. Similarly, Kieffer et al. reported that the abundance of various beneficial bacterial families (including *Lactobacillaceae*) showed negative correlations with liver nitrogenous metabolites (*e.g.*, amino acids, urea) in mice fed a high-fat diet and supplemented with resistant starch [45]. Altogether, these results seem to indicate that F treatment in CAF-fed rats could impact amino acid metabolism by modulating the microbiota composition and by decreasing the proteolysis rate. Supporting this hypothesis, transcriptomic data in PBMCs also revealed the downregulation of several genes involved in the proteasome in F-treated animals (Table S1). These results concur with previous studies reporting that obesity is associated with an increase in skeletal muscle atrophy and a decrease in myofibrillar proteins as a result of ubiquitin-mediated overactivation of the proteasome pathway [50], whereas F treatment could decrease the muscle atrophy caused by the CAF diet. Furthermore, this altered protein turnover was also reflected in a higher serum creatine:creatinine ratio in fiber-treated CAF-fed animals (Table 2), in line with previous works [51].

As expected, serum levels of 1-methylhistidine and 3-methylhistidine increased in CAF-fed animals compared with the STD group, as methylhistidine metabolites are well-known biomarkers of meat intake (*e.g.*, pâté and bacon supplied in the CAF diet) [28]. However, this increase was surprisingly sharper in response to the F treatment in the CAF group. Similarly, Korsholm et al. found that resveratrol treatment in men with MetS induced the accumulation of 3-methylhistidine and N-acetyl-3-methylhistidine in blood samples [52]. In particular, we observed that 1-methylhistidine concentrations correlated negatively with adiponectin levels and positively with liver weight in CAF-fed animals, whereas 3-methylhistidine negatively correlated with EWAT in STD animals (Fig. 3), which evidences the great impact of these amino acid derivatives on adiposity and liver health. Accordingly, deeper studies are needed to determine the exact effect of fibre on skeletal muscle function.

3.8. Impact of F on the crosstalk between circulating amino acids and intestinal health

The intake of fibres induced a significant decrease of serum and urinary levels of phenylalanine (*i.e.*, phenyllactic acid, phenylacetylglycine, phenylacetylglutamine) and tryptophan (*i.e.*, indolepropionic acid) derivatives in CAF-fed animals (Table 2, $P<.05$), in line with previously published studies [45,49,53,54]. These are common host-microbial co-metabolites produced by specific bacterial strains most of which are well-known uremic toxins. Our metagenomic results did not show any significant change in bacterial species that are recognized to be involved in aromatic amino acid metabolism, nor significant correlations between metabolomic and metagenomic data. However, serum amino acids and derivatives (*e.g.*, proline, phenyllactic acid) were positively associated with plasma LPS and negatively correlated with cecum weight in the CAF group (Fig. 3), thus corroborating the pivotal role of the interplay between the gut microbiota and amino acids in modulating endotoxemia and intestinal health.

Ergothioneine is a histidine metabolite that is usually acquired from the diet and shows antioxidant properties. Interestingly, we observed that the CAF diet lowered ergothioneine concentrations both in urine and serum samples, whereas F treatment tended to restore the original content of this compound in urine ($P=.037$, Table 2). Nonetheless, the mechanisms behind these metabolomic changes and consequences for health outcomes are uncertain, since no significant correlations were noticed with other biometrical, biochemical or metagenomic variables.

4. Conclusions

In conclusion, the results of our study demonstrate that supplementation over 8 weeks with a mixture of highly fermentable fibers at a dose extrapolable to humans reduced the adiposity of animals, improved intestinal health and had a clear effect on endotoxemia mainly in CAF-fed animals. The treatment also decreased liver cholesterol levels in STD-fed animals and reduced HbA1c blood levels in both STD- and CAF-fed animals. The integrative approach applied in this study evidenced that the major drivers in defining phenotypic status were biometrical and biochemical variables together with metabolomic data. Furthermore, correlation results suggest that the effect of F on obesity could be mediated by an increase of energy expenditure and lipid oxidation. Gene expression analysis in PBMCs is in accordance with some of the beneficial effects observed by the F treatment, showing significant changes in thermogenic, tight junction and proteolysis pathways. Metabolomics results evidenced an accumulation of urinary phenolic compounds and short-chain fatty acids as a consequence of F supplementation, as well as decreased levels of amino acids in serum and urine. This reinforces the central involvement of the gut microbiota and protein metabolism in the F-mediated ameliorating effects on the CAF-fed animals. Altogether, the present study highlights the added value of using integrative multi-omics approaches with the aim of obtaining a deeper understanding of the phenotypic effects and the underlying molecular mechanisms behind the beneficial effects of dietary interventions against obesity and related disorders. Further randomized controlled clinical trials focused on the effectiveness of F against obesity and its metabolic-related disorders would strongly contribute to shedding more light on this issue.

Author contributions

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Acknowledgments

We thank Juan María Alcaide, Yaiza Tobajas, Iris Triguero, Anna Antolin, Cristina Egea and Gertruda Chomiciute laboratory technicians at the Nutrition and Health Unit (Eurecat, Reus, Spain) for her valuable assistance with the animal studies, and Dr. Olga Jaúregui, Isidre Casals and Alberto Adeva from the Scientific and Technological Center of the University of Barcelona (CCiTUB), for their technical assistance with the targeted Metabolomic Platform.

Declarations of competing interests

J.A.M.-M., J.J. and M.M.-P. are employees of Laboratorios Ordesa, Barcelona, Spain. The rest of the authors have no known conflicts of interest associated with this publication.

Funding

This work was financially supported by the Centre for the Development of Industrial Technology (CDTI) of the Spanish Ministry of Science and Innovation under grant agreement: SMARTFOODS project (IDI-20141206). C.A.-L. gratefully acknowledges the CIBERfes from ISCIII co-funded by the FEDER Program from the European Union, "A way to make Europe"; the Generalitat de Catalunya's Agency AGAUR (2017 SGR1546) and ICREA Academia Award 2018. R.G.-D. thanks the "Juan de la Cierva" program from MINECO (IJC2019-041867-I).

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jnutbio.2022.109184.

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A mixture of four dietary fibres ameliorates adiposity and improves metabolic profile and intestinal health in cafeteria-fed obese rats: an integrative multi-omics approach

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Material and methodologies

Serum, liver and colon biochemical analysis

Enzymatic colorimetric kits were used to determine glucose, triglycerides, total cholesterol (QCA, Barcelona, Spain) and non-esterified fatty acids (NEFAs) (FUJIFILM Wako Chemicals Europe, Neuss, Germany). Fluorometric kits from BioAssay Systems (California, United States) were used for HDL and LDL cholesterol determination.

ELISA kits were used for the determination of leptin (Millipore, Barcelona, Spain), adiponectin (Millipore, Barcelona, Spain), glycosylated haemoglobin (HbA1c) (MyBiosource, Madrid, Spain), monocyte chemoattractant protein 1 (MCP-1) (Thermo Scientific, Madrid, Spain), apolipoprotein B-100 (Cusabio, Vizcaya, Spain), oxidized LDL (Cusabio, Vizcaya, Spain) and thiobarbituric acid reactive substances (TBARS) (Cayman Chemical, Barcelona, Spain).

The circulating levels of lipopolysaccharides (LPS) were determined by a method based on the alkaline hydrolysis of serum samples, the derivation of the 3-hydroxy fatty acids (3OHFAs) obtained and their subsequent quantification by gas chromatography coupled to mass spectrometry (GC-MS) [1] (Agilent technologies, California, United States).

The liver lipid content was analysed using previously established protocols [2]. The lipids were quantified using colorimetric kits for triglyceride and total cholesterol (QCA, Barcelona, Spain).

Myeloperoxidase (MPO) activity was analysed in the colon using a protocol described previously [3].

Metagenomics of caecum content

DNA was extracted from 200 mg of caecal content using a QIAmp DNA stool mini kit (Qiagen Inc., Hilden, Germany) according to the manufacturer's instructions. DNA purity and integrity were assessed using spectrophotometry (NanoDrop, Thermo Fisher Scientific, Massachusetts, USA).

16S rRNA gene amplification and purification was carried out using 50 ng of DNA and the AmpliTaq Gold 360 Master Mix (Applied Biosystems, Thermo Fisher Scientific, Massachusetts, USA) in a Veriti Thermal Cycler (Applied Biosystems, Thermo Fisher Scientific, Massachusets, USA). An Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, California, USA) and the associated Agilent DNA 7500 Reagent Kit (Agilent Technologies, Santa Clara, California, USA) were used to determine the quality, length and concentration of libraries needed for the sequencing procedure.

An Ion 520 and Ion 530 Kit-Chef (Life Technologies, Carlsbad, California, USA) were employed for template preparation and sequencing according to the manufacturer's instructions. Each mixture of prepared samples was loaded on a 530 chip and sequenced using the Ion S5 system

(Life Technologies, Carlsbad, California, USA). After sequencing, Ion Torrent Suite software removed low-quality and polyclonal sequences and those reads were then analysed using QIIME. The analysis included OTU (operational taxonomic unit) clustering, OTU analysis and species annotation. Metagenomics analysis was done at the Centre for Omics Sciences (Reus, Spain).

Transcriptomic analysis of peripheral blood mononuclear cells (PBMCs)

The RNA samples were extracted from PBMCs using the trizol:chloroform method and quantified using spectrophotometry (NanoDrop, Thermo Fisher Scientific, Massachusetts, USA). Their integrity was analysed with an RNA 2100 Bioanalyzer (Agilent Technologies, California, United States) with an RNA 6000 Nano Kit (Agilent Technologies, California, United States) and the protocol *Eucaryotic RNA Nano* in Agilent 2100 Expert software (Agilent Technologies, California, United States).

PBMC transcriptomics was performed using 50 ng of RNA with a SurePrint G3 Rat Gene Expression v2 8x60K Microarray (Agilent Technologies, G4858) and the *Agilent One-Color Microarray-Based Exon Analysis Low-Input Quick Amp WT Labeling Kit* protocol version 6.9.1 (Agilent Technologies, California, United States) was followed according to the manufacturer's instructions. Agilent Scan Control version A.8.5.1 software (Agilent Technologies, California, United States) was used to scan the slides with a 3 µm resolution with an Agilent G2565CA Microarray Scanner System with SureScan High Resolution Technology (Agilent Technologies, California, United States). The statistical analysis to find the significant changes between conditions included in the present study was done at gene level using Gene Spring GX software v. 14.9 (Agilent Technologies, California, United States). Transcriptomic analysis was done at the Centre for Omics Sciences (Reus, Spain).

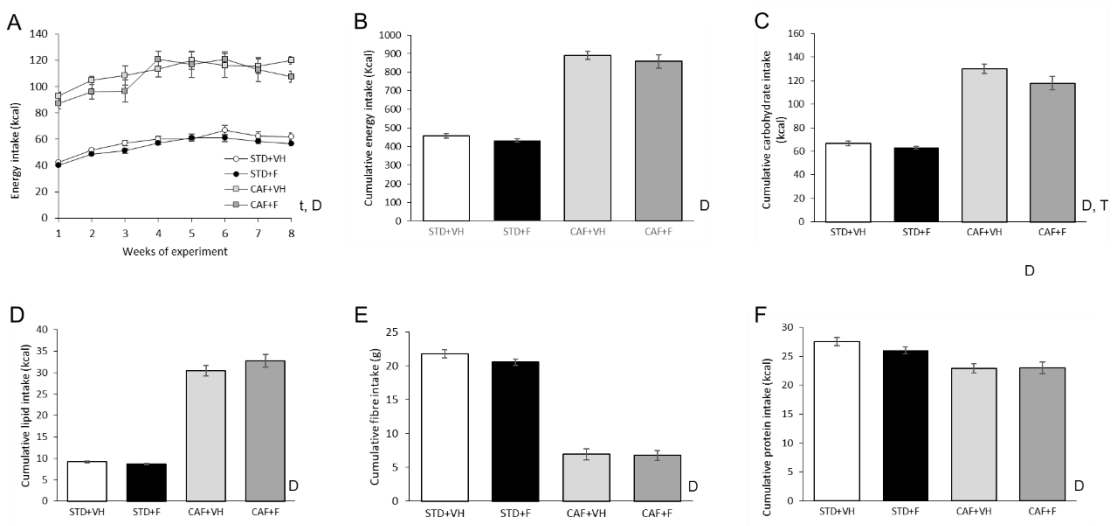
Metabolomic analysis of serum samples by ^{1}H-NMR

Serum extraction was performed using 150 ul of serum, adding 300 uL of methanol and 100 uL of water and vortexing vigorously. Then, 100 uL of chloroform was added and mixed using an orbital shaker at 300 rpm for 10 min. After that, 500 uL of chloroform and 200 ul of water were added again and centrifuged at 5000 g for 15 min at 4 ºC. The solution was separated into an upper methanol/water phase, which contains polar metabolites, and a lower chloroform phase, containing lipophilic compounds. Both phases were divided into separate glass vials and solvents were removed using a speed vacuum concentrator and then stored at -80 ºC. Lyophilization was required for some samples before NMR measurement. Metabolomic analysis was done at the Centre for Omics Sciences (Reus, Spain).

Metabolomic analysis of serum and urine samples by UHPLC-MS/MS

Multi-targeted quantitative metabolomic analyses of serum and urine samples were conducted by ultra-high-performance liquid chromatography coupled to tandem mass spectrometry (UHPLC-MS/MS), following the methodologies described elsewhere with slight modifications [4,5]. Briefly,

serum samples were subjected to protein precipitation by mixing 30 µL of each study sample with 200 µL of cold acetonitrile. The samples were vigorously vortexed for 1 min, kept at -20 ºC for 10 min, and finally centrifuged at 10000 g for 10 min at 4 ºC to transfer the supernatants to 96-well injection plates. On the other hand, pre-centrifuged urine samples were diluted tenfold with ultrapure water containing 0.1% formic acid and then transferred to the injection plates. Furthermore, urine samples were also subjected to solid-phase extraction (SPE) using Oasis® HLB extraction plates (Waters, Milford, MA, USA) with the aim of pre-concentrating minor low-polarity metabolites, especially those coming from dietary sources, as previously described [4]. A set of internal standards (100 µg L-1) was added to all the sample extracts for quantification. Afterwards, samples were analysed by UHPLC-MS/MS for high-throughput quantitative metabolomic fingerprinting, using the chromatographic and MS conditions optimized by González-Domínguez et al. [5].



Supplementary Figure 1. Food intake-related parameters of rats fed standard (STD) or cafeteria (CAF) diet and treated with vehicle (VH) or fibre (F). A: Energy intake. B: Cumulative energy intake. C: Cumulative carbohydrate intake. D: Cumulative lipid intake. E: Cumulative fibre intake. F: Cumulative protein intake. The data are presented as the mean ± SEM. D, the effect of the diet; T, the effect of the fibre treatment; t, the effect of the time; DxT, the interaction between diet and treatment (Two-way ANOVA and RMs ANOVA, $p < 0.05$).

Supplementary Table 1. Significantly changed metabolic pathways from KEEF database based on 200 gene analysis between cafeteria diet-fed group treated with vehicle (CAF+VH) and cafeteria diet-fed group treated with fibre (CAF+F).

Genes (EntrezGeneID)	Process Name	Num_Genes	Gene_group	Percentage (%)	*P value*	Benjamini and Hochberg (FDR)
25581; 288455; 289924; 29614; 29672; 408248; 58854;	rno03050~Proteasome	7	48	14.6	2.35E-007	3.06E-005
100360682; 290519; 298385; 361808; 681031;	rno03040~Spliceosome	5	138	3.6	0.0054	0.3456
293130; 293508; 293991; 29754; 81728;	rno05012~Parkinson_disease	5	152	3.3	0.0080	0.3456
293130; 293508; 293991; 29754; 501560; 81728;	rno04714~Thermogenesis	6	243	2.5	0.0139	0.4441
25700; 293130; 293991; 29754; 81728;	rno05010~Alzheimer_disease	5	185	2.7	0.0171	0.4441
362706; 690945;	rno00030~Pentose_phosphate_pathway	2	31	6.5	0.0275	0.4906
293130; 293991; 29754; 81728;	rno00190~Oxidative_phosphorylation	4	143	2.8	0.0286	0.4906
289323; 299198; 317579;	rno03008~Ribosome_biogenesis_in_eukaryotes	3	89	3.4	0.0358	0.4906
293130; 293508; 293991; 81728;	rno04723~Retrograde_endocannabinoid_signaling	4	155	2.6	0.0366	0.4906
293508; 498030; 501560;	rno04914~Progesterone-mediated_oocyte_maturation	3	91	3.3	0.0377	0.4906
293508; 315655; 84006; 84588;	rno04530~Tight_junction	4	170	2.4	0.0483	0.5705

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9.2 Conferences

Poster 1

Title: Nutrimetabolomic study to identify biomarkers of anthocyanins: Targeting the gut microbiota activity through a randomized, controlled, cross-over trial in healthy individuals.

Authors: **Mostafa H.**, Meroño T., Gonzalez-Dominguez R., Rudloff S., Kuntz S., Andres-Lacueva, C.

Conference:

- The 10th International Conference of Polyphenols and Health (ICPH). London, United Kingdom, 2022.
- The 18th International Conference of the Metabolomics Society. Valencia, Spain, 2022.

Nutrimetabolomic study to identify biomarkers of anthocyanins: Targeting the gut microbiota activity through a randomized, controlled, cross-over trial in healthy individuals

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NutriMetabolom

nutrimetabolomics

INTRODUCTION / OBJECTIVES

- Following the consumption of an ACN-rich juice, plasmatic metabolites isolated from human plasma inhibited the in-vitro migration of pancreatic cancer cells (1,2).
- Due to extensive microbial metabolism of ACN in the colon (**Figure 1**), the identity of the metabolites behind such effects remains unknown (1).

Objective:

The ATTACH project aims to identify the gut microbiota metabolites associated with the anti-proliferative and anti-migratory properties in pancreatic and colon cancer cell lines.

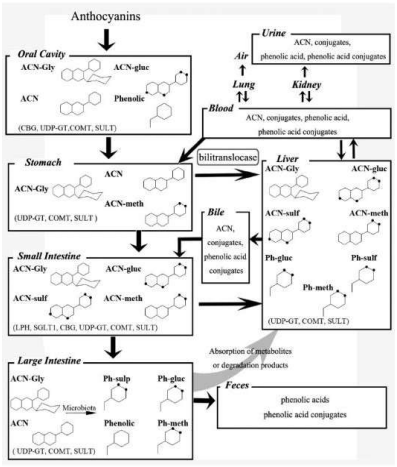

Figure 1. ACN metabolism (Taken from (3))

METHODS

Crossover design trial: Participants were randomly assigned to 2 cycles of the intervention (28d) getting the ACN-rich juice (verum) or ACN-depleted placebo. After a 7-day run-in period, the 28d intervention period followed. After the intervention period, a 7-day run-out phase completed the first cycle. Before (day 0) and after (day 28) intervention, fecal, blood and 24-h-urine samples were collected and processed for targeted metabolomics analysis as shown in **Figure 2**.

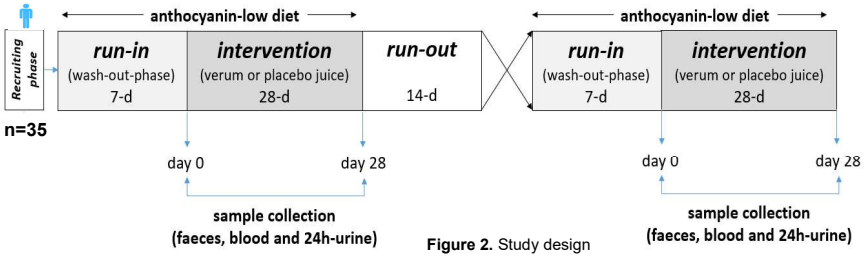

Figure 2. Study design

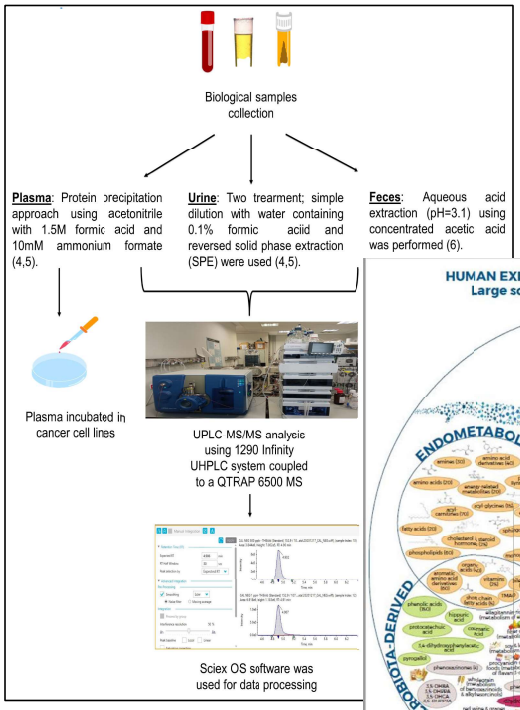

Figure 3. Targeted metabolomics workflow (adapted from (5))

Experimental procedure:

The targeted metabolomics UHPLC-MS/MS method (Sciex QTRAP 6500) for large-scale quantitative exposome research in plasma, urine and faeces samples was carried out. The coverage of this method comprises about 450 food-derived metabolites including polyphenol metabolites produced by gut microbiota (like valerolactones, and phenolic acids), **Figure 3 and 4**.

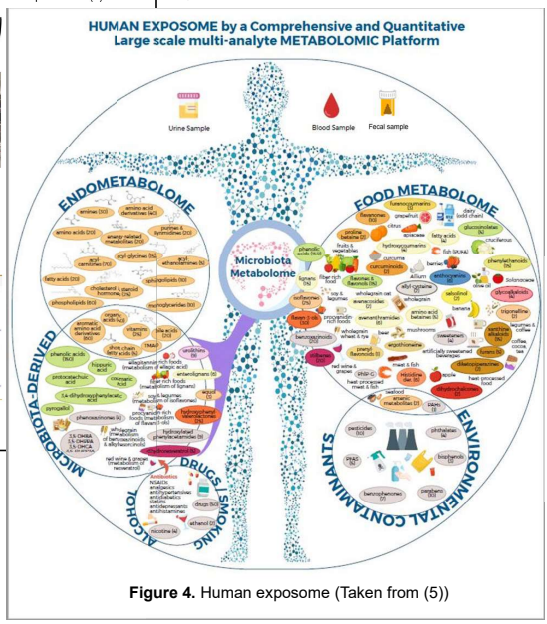

Figure 4. Human exposome (Taken from (5))

RESULTS / FINDINGS

From plasma, urine and fecal metabolome, 22 metabolites from the 1,232 metabolites including endogenous, exogenous and phase II metabolites that were evaluated were significantly associated with ACN-rich juice intake as shown in the volcano plot, **Figure 5**.

Table 1. Characteristics of the study population

Covariates	Volunteers (n=35)
Age (years) mean (SD)	24.5 (2.5)
Gender n (%) Female Male	27 (77%) 8 (23%)
BMI (Kg/m^2) mean (SD)	21.7 (2.3)

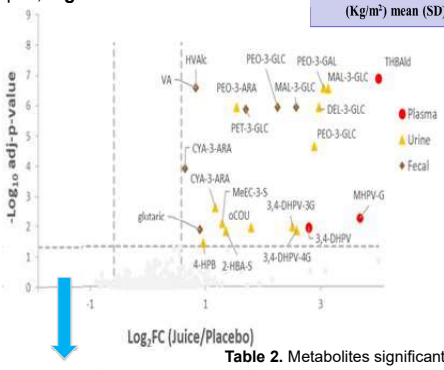

Figure 5. Volcano plot of log2FC vs log10 FDR-adjusted p-value, showing the metabolites that are significantly associated with ACN intake in plasma, urine and feces. The FC value of each metabolite corresponds to the difference between log2FCpost/pre during ACN-rich juice and log2FCpost/pre during placebo intervention

Table 2. Metabolites significantly associated with ACN intake

	Parent ACN	Phenolic compounds	Amino acid derivatives
Plasma	-	1. 2,4,6-trihycroxybenzaldehyde (THBAld) 2. 4'-hydroxy-3'-methoxyphenyl-γ-valerolactone glucuronide (MHPV-G) 3. 3',4'-dihydroxyphenyl-γ-valerolactone (3,4-DHPV)	-
Urine	1. Cyanidin 3-arabinoside 2. Peonidin 3-arabinoside 3. Peonidin 3-glucoside 4. Peonidin 3-galactoside 5. Malvidin 3-glucoside 6. delphinidin 3-galactoside	1. O-coummaric acid (oCOU) 2. 4-hydroxybenzophenone (4-HPB) 3. 3'-methylepicatechin sulfate (3-MeEC-S) 4. 2-hydroxybenzoic acid sulfate (2-HBA-S) 5. 3',4'-dihydroxyphenyl-γ-valerolactone 3'-glucuronide (3,4-DHPV-3G) 6. 3',4'-dihydroxyphenyl-γ-valerolactone 4'-glucuronide (3,4-DHPV-4G)	-
Feces	1. Cyanidin 3-arabinoside 2. Peonidin 3-glucoside 3. Petunidin 3-glucoside 4. Malvidin 3-glucoside	1. Vanillic acid (VA) 2. Homovanillyl alcohol (HVAlc)	1. Glutaric acid

CONCLUSIONS

- Gut microbiota phenolic metabolites are largely increased in biological samples in comparison to parent ACN
- Plasma biomarkers of ACN intake as THBAld, MHPV-G and 3,4-DHPV might be responsible for some of the health benefits associated with ACN consumption such as anti-cancer activities.

ACKNOWLEDGEMENTS / FUNDING

We are deeply grateful to all the volunteers who participated in the study.
The study was funded by the Deutsche Forschungsgemeinschaft (DFG) (grant KU1925/4-1).
This study was further supported by CIBERFES, funded by Instituto de Salud Carlos III and co-funded by European Regional Development Fund "A way to make Europe" and the award of the Generalitat de Catalunya's Agency AGAUR [2017SGR1546] and ICREA Academia 2018. Hamza would like to thank the scholarship *ajuts de personal investigador predoctoral en formació (APIF)* from the University of Barcelona.

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Poster 2

Title: Integrative Multi-Omics Approach:Health Effects Of A Mixture Of Dietary Fibres In Obese Rats

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Conference: DDLS Annual Conference. Stockholm, Sweden, 2022.

INTEGRATIVE MULTI-OMICS APPROACH:
HEALTH EFFECTS OF A MIXTURE OF DIETARY FIBRES IN OBESE RATS

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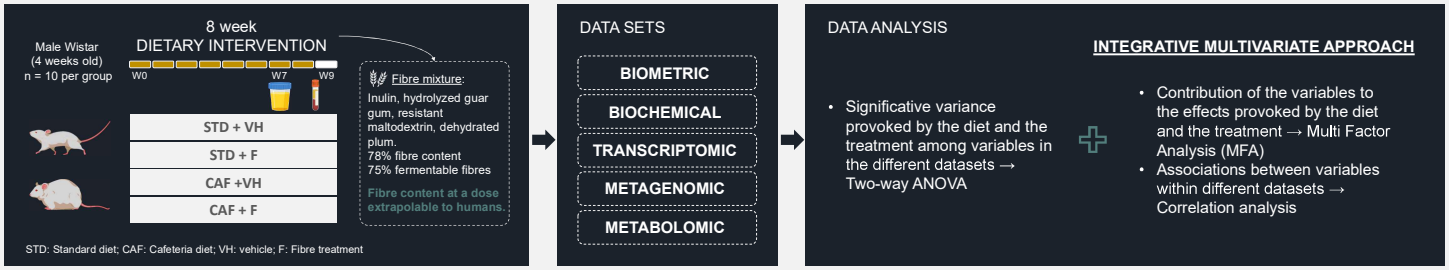
BACKGROUND

Metabolic syndrome is a pathologic condition composed of a constellation of health risk factors such as insulin resistance, central obesity, systemic hypertension, and atherogenic dyslipidemia; which could lead to increased **risk for many cardiovascular diseases**. It is a major health hazard worldwide and a leading cause of mortality an morbidity.
Diet is among the most important modifiable behaviors for the prevention and amelioration of these disorders. **Fibre intake is presented as an strategy to improve metabolic profile and disorders associated to metabolic syndrome**.
In such multifactorial disorders it is fundamental to combine **multidimensional data to get a comprehensive picture** of the effects elicited by dietary interventions.

OBJECTIVE

Study the **effect of a dietary fibre mixture intake on an obese metabolic phenotype** and its health outcomes, by using **integrative multi-omics, biometrical and biochemical approaches** to obtain a **deeper understanding** of the phenotypic effects and the underlying molecular mechanisms.

METHODS



RESULTS

The integrative multi-omics approach highlights the potential of supplementation with a mixture of fibres to ameliorate the impairments triggered by obesity in terms of adiposity, metabolic profile, and intestinal health. The intervention effect was reflected at the metabolomic, metagenomic and transcriptomic levels.

Fibre treatment:
↓ significant reduction in body weight, adiposity, HbA1c and HDL-cholesterol serum levels, and colon MPO activity,
↑ increase in caecal weight and small intestine length:weight ratio.
Metabolomics: ↑ increase of urinary phenolic compounds and short-chain fatty acids. ↓ decreased levels of amino acids in serum and urine.
Transcriptomics: ✓significant beneficial changes in thermogenic, tight junction and proteolysis pathways.

CAF+F group:
↑ significant enhancement in energy expenditure, fat oxidation and fresh stool weight,
↓ significant reduction in adiponectin and LPS serum levels, compared to control group.

STD+F group:
↓ reduced serum LDL-cholesterol levels and a significant reduction in total cholesterol levels in the liver compared to STF+VH group.

MFA modelling: First dimension: Clear differentiation between CAF and STD groups; Second dimension: separation of F-fed animals, mainly in the CAF group (Fig. 1A). Overall this could be understood as an adaptative mechanism to counteract the alteration of energy homeostasis produced by CAF consumption. The major drivers in defining phenotypic status were biometrical and biochemical variables together with metabolomic data (Fig. 1B).

Correlation analysis: This integrative multi-omics analysis enabled us to comprehensively elucidate the molecular mechanisms and biological pathways influenced by the F intervention.

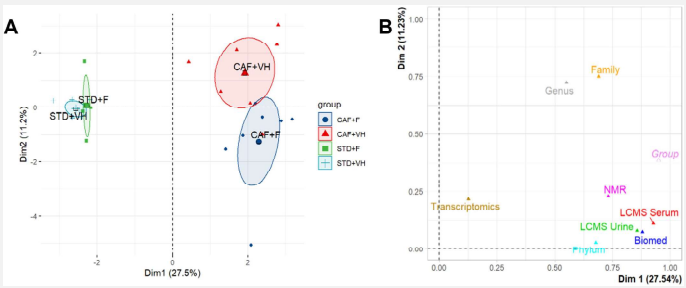
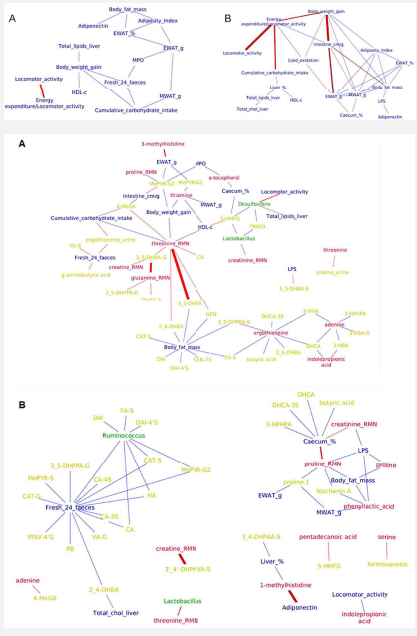


Fig. 1. Summary of multiple factor analysis (MFA) results. Scatter plots showing the projection of individual animals from the four study groups in the space defined by the first two dimensions, using the significant variables according to two-way ANOVA (A). Variable representation plots showing the projection of the different data in the space defined by the first two dimensions, using the significant variables according to two-way ANOVA (B).



Figs. 2-3. Network representation of the associations between biometrical/biochemical parameters (Fig. 2) and metabolomic, metagenomic and biometrical/biochemical variables (Fig. 3), as a result of F treatment in animals fed the STD diet (A) and the CAF diet (B). Pearson correlations were computed on significant variables and significant correlations (P-value ≤.01) were represented. Positive correlations are shown in blue and negative correlations in red. The thickness of the line refers to the value of the correlation, the correlation being greater the thicker the line. Label colors differentiate the different sets of measures: blue for biometrical/biochemical parameters; red for serum metabolomics; yellow for urine metabolomics; green for metagenomics.

Take-home messages:

Added value of using integrative multi-omics approaches with the aim of obtaining a deeper understanding of the phenotypic effects and the underlying molecular mechanisms behind the beneficial effects of dietary interventions against obesity and related disorders.

✓ Fibre intake reduced body weight and adiposity.
✓ Enhancement of energy expenditure and lipid oxidation by fibre intake.
✓ Fibre intake improved intestinal health and endotoxaemia.
✓ Involvement of gut microbiota and protein metabolism in the effects observed.

Núria Estanyol-Torres, Cristina Domenech-Coca, Raúl González-Domínguez, Antonio Miñarro, Ferran Reverter, Jose Antonio Moreno-Muñoz, Jesús Jiménez, Manel Martín-Palomas, Pol Castellano-Escuder, Hamza Mostafa, Santi García-Vallvé, Nerea Abasolo, Miguel A. Rodríguez, Helena Torrell, Josep M del Bas, Alex Sanchez-Pla, Antoni Caimari, Anna Mas-Capdevila, Cristina Andres-Lacueva, Anna Crescenti. A mixture of four dietary fibres ameliorates adiposity and improves metabolic profile and intestinal health in cafeteria-fed obese rats: an integrative multi-omics approach. The Journal of Nutritional Biochemistry, Volume 111, 2023, 109184, ISSN 0955-2863, https://doi.org/10.1016/j.jnutbio.2022.109184.

This work was financially supported by the Centre for the Development of Industrial Technology (CDTI) of the Spanish Ministry of Science and Innovation under grant agreement: SMARTFOODS project (IDI-20141206). C.A.-L. gratefully acknowledges the CIBERfes from ISCIII co-funded by the FEDER Program from the European Union, "A way to make Europe"; the Generalitat de Catalunya's Agency AGAUR (2017 SGR1546) and ICREA Academia Award 2018. R.G.-D. thanks the "Juan de la Cierva" program from MINECO (IJC2019-041867-I).