Thesis

Metabolomics quality: preanalytical short-storage impact with regard to heterogenous factors

A multidisciplinary team study to detect metabolomics changes in blood samples after short-storage compared with immediate centrifugation and overview on pleiotropic factors in view of preanalytical short-storage quality.

Submitted by

Abdullah Al-Baghdadi

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Medical University of Graz
Department of Internal Medicine
Division of Endocrinology & Diabetology
Centre for Biomarker Research in Medicine CBmed GmbH

First and second Supervisors:

Univ.-Prof.^{in} Dr.^{in} med. univ. Barbara Obermayer-Pietsch
Dr.^{in} Natalie Bordag

Contributor:

Dr.^{in} Eva Svehlikova

Graz, 29.06.2022

Declaration

I hereby declare that I have authored this thesis independently, that I have not used other than the declared sources / resources, and that I have explicitly marked all material which has been quoted either literally or by content from used sources.

This study was conducted and designed with the Declaration of Helsinki and was reviewed by the ethical committee of the Medical University of Graz, Austria (31-116 ex 18/19, 16.01.2019). Prior to initiation of any study activities, a written informed consent from all participants was obtained.

Graz, 29.06.2022 *Abdullah Al-Baghdadi eh.*

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والاهم، ان كل هذا لم يكن ليحدث لولا -بفضل الله- عائلتي: امي التي كانت تذكرني صباحا ومساءا، في كل دعاء وابتسامة، وأبي الذي علمني مواجهة هذه الحياة ودعمني في حياتي الخاصة والاكاديمية، اخواتي واخوتي، أعمامي وعماتي، اخوالي وخالاتي، وزوجتي الوفية، هذه الاطروحة هي شهادة على دعمكم وحبكم غير المشروط.

Dedication

To the grand Ayatullah Seyed Ali Al-Sistani, the light in our darkness, and to my greater family, the inspiring team of Al-Ayn social care foundation around the world, for the delightful help they provide to empower the widows and orphaned children, especially in Iraq. I dedicate this humble work.

الى النور في ظلماتنا ، سماحة آية الله العظمى السيد علي السيستاني –دام ظله–

والى عائلتي الأكبر، فريق مؤسسة العين للرعاية الاجتماعية الملهم في كل العالم

لما يقدموه من عمل منقطع النظير في دعم ورعاية الارامل واليتامى

لاسيما في العراق الحبيب . .

اهدي هذا العمل القليل .

Contents

Abbreviations

Table 1 Abbreviations

PCs	phosphatidylcholines
SMs	sphingomyelins
LPCs	lysophosphatidylcholines
AA	amino acid
AC	acylcarnitine
BCAA	branched-chain amino acid
PE	phosphatidylethanolamine
PC	Phosphatidylcholine
CE	cholesterol esters
GPE	glycero-3-phosphoethanolamine
GPI	glycerophosphoinositol
SCN	suprachiasmatic nucleus
MS	mass spectrometry
PESI-MS	probe electrospray ionization- Mass-spectrometry
CVD	cardiovascular disease
T2DM	type 2 diabetes mellitus
IVF	in vitro fertilization
CT-Scan	computed tomography scan
NMR	nuclear magnetic resonance
FDR	false discovery rate
SOPs	standard operating procedures
GPE	glycero-3-phosphoethanolamine
GPI	glycerophosphoinositol
PLS-DA	partial least squares discriminant analysis

Figures and tables

Abstract

Introduction: Metabolomics is a tool for understanding pathophysiological progressions and finding prognostic biomarkers of various disorders to achieve an early and accurate diagnosis and treatment. However, a comprehensive understanding of metabolomics is challenged by understanding the factors that may impact the state of metabolite. Most importantly, errors in the blood sampling stage can potentially lead to significant misleading laboratory results, including metabolomics results. More specifically, preanalytical short storage errors are amongst the most common analytical errors. They can produce misleading results which lead to patient harm. Furthermore, a robust, practical, and economically viable measurement tool is indispensable for ensuring the sample quality. We conducted the present study in order to predict any metabolomics changes after preanalytical short storage with regard to other intrinsic and extrinsic factors. We used a new quality control technique for this purpose.

Method: We recruited 50 participants to two groups. The first group, the homogenous group, had minimum biological variations. The second group, the heterogeneous group, had at least one biological variation. Dual samples from each participant were obtained, a control sample was centrifuged immediately, and a case sample was left at room temperature for three hours prior to centrifugation. The samples were analyzed with PESI-MS.

Result: Our results showed that out of 1200 statistically valid features, the vast majority were stable after the short storage period. Ten analytes were found to be significantly changed after three hours storage. Furthermore, no significant difference was found between the two groups (homogeneous, heterogeneous).

Conclusion: Taking into account the potential impact of intrinsic and extrinsic factors on metabolomics results, our results have shown that PESI-MS is a sufficiently robust method to capture short-term storage of samples. This underlines the potential of the PESI-MS method as a new approach to quality control of blood samples.

Summery (German)

Einleitung: „Metabolomics“ sind ein technisches Diagnostikinstrument, um pathophysiologische Verläufe zu verstehen und prognostische Biomarker für verschiedene Erkrankungen zu finden, um eine frühe und genaue Diagnose und Behandlungsentscheidung erreichen zu können. Ein umfassendes Verständnis von Metabolomics wird jedoch durch das Verständnis von verschiedenen Einflussfaktoren erschwert, die die Ergebnisse verändern können. Am wichtigsten ist, dass Fehler bei der Blutentnahme potenziell zu erheblich irreführenden Laborergebnissen führen können. Insbesondere gehören präanalytische Fehler bei einer kurzen Lagerung zu den häufigsten analytischen Problemen. Darüber hinaus ist ein robustes, praktisches und wirtschaftlich tragfähiges Messinstrument unerlässlich, um die Qualität der Proben sicherzustellen. Wir haben die vorliegende Studie durchgeführt, um etwaige metabolomische Veränderungen nach präanalytischer Kurzlagerung im Hinblick auf andere intrinsische und extrinsische Faktoren vorherzusagen. Zu diesem Zweck wurde eine neue Technik zur Qualitätskontrolle eingesetzt.

Methode: Wir haben 50 Teilnehmer*innen für zwei Gruppen rekrutiert. Die erste (homogene) Gruppe, wies ein Minimum an biologischen Variationen auf. Die zweite (heterogene) Gruppe, zeigte mindestens eine biologische Abweichung. Von jeder/m Teilnehmer*in wurden zwei Proben entnommen, eine Kontrollprobe wurde sofort zentrifugiert und eine Fallprobe wurde vor der Zentrifugation drei Stunden lang bei Raumtemperatur gelagert. Die Proben wurden mit PESI-MS analysiert.

Ergebnis: Unsere Ergebnisse zeigten, dass von den 1200 statistisch gültigen Merkmalen die überwiegende Mehrheit nach einer kurzen Lagerzeit stabil war. Zehn Metabolomics-Analyten wurden signifikant durch die 3 h Lagerung verändert. Außerdem wurde kein signifikanter Unterschied zwischen den beiden Gruppen (homogen, heterogen) festgestellt.

Schlussfolgerung: Unsere Ergebnisse haben unter Beachtung der potenziellen Auswirkungen von intrinsischen und extrinsischen Faktoren auf Metabolomics-Ergebnisse gezeigt, dass PESI-MS eine ausreichend robuste Methode um die Kurzzeitlagerung von Proben zu erfassen. Dies unterstreicht das Potential der PESI-MS Methode als neuen Ansatz zur Qualitätskontrolle von Blutproben.

Publications from this study

Peer-reviewed:

Blood Plasma Quality Control by Plasma Glutathione Status (Tomin, Bordag *et al.*, 2021)

Preprint:

Towards fast, routine blood sample quality evaluation by Probe Electrospray Ionization (PESI) metabolomics (Bordag *et al.*, 2021)

1 INTRODUCTION

“Metabolism” is the summary of basic biochemical reactions that drive vital cellular functions to sustain the life of the organisms (Judge and Dodd, 2020). The intermediate or final results of these reactions are low-molecular-weight (<1500 Da) molecules termed metabolites. The quantitative and qualitative collection of the metabolites is termed the metabolome, which includes, for example, fatty acids, carbohydrates, amino acids and lipids (Færgestad *et al.*, 2009), while the analytical and profiling technique of the metabolites is termed metabolomics (Manchester and Anand, 2017).

In a clinical setting, the ultimate goal of metabolomics is to have a robust and clear understanding of the biological changes (Johnson, Ivanisevic and Siuzdak, 2016). This may help us to accomplish a better understanding of the pathophysiological progressions and to find prognostic biomarkers of various diseases and disorders in order to achieve an early and accurate diagnosis. This will also help to find an appropriate treatment depending on the bio-physiological state of the patient. Furthermore, it is to be used as an early and focused monitor of the body's response for the therapy up to the point of predicting survival (Johnson and Gonzalez, 2012; Bujak *et al.*, 2015).

A comprehensive understanding of metabolomics is challenged and limited principally by understanding all, or at least most, of the factors which potentially may impact the state of the metabolomes (Johnson and Gonzalez, 2012). These factors are usually divided into intrinsic and extrinsic factors. Some of these are due to inherited abnormalities or changes, whilst others are due to environmental or iatrogenic factors (Johnson *et al.*, 2012). Amongst these factors, iatrogenic factors, in particular, sampling and laboratory errors, are the most common factors which may impact the metabolomics and produce misleading results (Johnson and Gonzalez, 2012).

At this point, it is critical to define the errors, to understand their effect, and to ensure standardization, which may help to minimize or limit the undesirable impact of the misleading results. It therefore becomes necessary as a first step to try to understand their impact in clinical settings. The ultimate value in carrying out this research will lie in the benefit to the patient and management of their condition. This will be discussed in section on analytical errors and patient management (1.2).

1.1 Metabolomics methods

There are several techniques used in metabolomics analysis, including mass-spectrometry (MS), which is one of the most widely used techniques in the field. Mass-spectrometry (MS) is a sensitive analytic technique in metabolomics to identify chemical substances based on their mass-to-charge ratios (Brown, 2020). Chromatography is used coupled to MS in metabolomics analysis to achieve a better separation of metabolites with agood chemical coverage (Sands et al., 2021). Chromatography is a physical procedure used for separating the components of a gas or liquid mixture (Giddings, Calvin & Keller, 2021). The downside of such metabolomics set-ups is the large demand for highly specialized expertise in this technique. In addition, the procedures needed to achieve successful, high-quality research are expensive and complicated (Segers et al., 2019).

In contrast, probe electrospray ionization-mass-spectrometry (PESI-MS) omits the chromatography and captures a small sample droplet by rapidly dipping a needle tip directly into the sample. Next a high voltage under ambient conditions is applied to the needle to generate an electrospray (Hiraoka et al., 2007). This simplifies and shortens the handling time to minutes (Hiraoka et al., 2020), allowing for a robust and practical, economically viable measurement tool.

1.2 Analytical errors and patient management

Laboratory tests in general have been an indispensable part of medical care for decades in the management of most clinical health disorders. They are essential for the management from the start until the end, and they include the following: screening, diagnosis, treatment, and follow up. Statistically, up to 80-90% of the medical diagnoses of physicians are based on, or made in conjunction with, laboratory tests (Agarwal, 2013). The accuracy of the laboratory tests therefore becomes vital. It then becomes necessary to work with every stage of the blood sampling and analysis to make it as accurate as possible in order to avoid any potential factors that may influence the results.

Blood analysis errors usually break into three types depending on the stage in which they occur: pre-analytical, analytical, and post-analytical errors. Amongst the three, pre-analytical errors are considered the most common (Plebani *et al.*, 2014). It therefore becomes highly important to prioritize the standardisation of blood sampling and pre-analysis procedures.

The importance of the pre-analytical errors in routine blood sampling is that they are frequent but often overlooked in routine clinical practice. Unfortunately, such errors have taken place despite the existence of standard operating procedures (SOPs) for the blood sampling and plasma/serum preparation. It is a notable finding by Salvagno et al. (2008) that 5.5% of inpatient routine diagnostic samples were unsuitable for further processing due to multiple errors. Sample haemolysis, clotting, and inappropriate volume were recorded in the following percentages respectively: 19.5%, 14.2%, and 13.7%.

Pre-analytical errors can also result in undermining the quality of laboratory tests, and they can lead to a considerable impact on patient management. For example, 30% of laboratory errors have led to unnecessary further laboratory tests, as well as to further invasive and non-invasive procedures, such as vascular interventions, CT-Scans, nuclear magnetic resonance (NMR), biopsies, and additional consultations (Plebani, 2006). Such unnecessary diagnostic procedures result in excessive costs, further undesirable discomfort for patients, and they place pressure on the limited time and personnel resource shortages in the health care system.

Numerous pre-analytical procedures on blood samples can affect metabolomics. A number of these procedures are usually controllable, like temperature and storage materials. There are other procedures which are uncontrolled due to various reasons, such as pre-analytical short-storage; particularly when there is a missing concrete protocol to control it.

With regard to uncontrolled procedures, short-storage for up to three hours at room temperature is the most common, compared with long-storage in other environments (Yin *et al.*, 2013; Yin, Lehmann and Xu, 2015). As short-storage is the most common uncontrolled procedure, it has therefore become the focus of this study. The impact of pre-analytical short storage on metabolomics quality will be discussed in the following sections. This may lead to development of a concrete protocol. We can leave the other types of errors for future studies to cover.

1.3 Impact of pre-analytical short-storage at room temperature

The pre-analytical period of blood samples can be prolonged at room temperature (RT) for many reasons. This may impact billions of active metabolites and lead to misleading changes in metabolomics (Yin, Lehmann and Xu, 2015). The components of the metabolome are significantly affected by prolonged coagulation processes. And this information is derived from nuclear magnetic resonance technologies (NMR) (Griffin *et al.*, 2011). Additional studies have confirmed that changes are seen in concentrations of many molecules, such as alanine transaminase and glucose. These changes are observed even when anticoagulants have been used, such as ethylenediamine tetra acetic acid (EDTA) (Clark *et al.*, 2003; Bruns and Knowler, 2009; Wong *et al.*, 2013). This effect was also demonstrated by a study on 803 plasma samples. The study reported that a total of 149 plasma analytes changed in different directions during a delay of plasma separation from 30 minutes to four days. Furthermore, some changes were seen in just two to four hours pre-analysis. Analytes contents were seen to increase up to two-fold in some metabolomes, such as inosine, glutamate, malate, and adenosine monophosphate.

In contrast, others were decreased up to two-fold, such as, 1-methylguanosine, arginine and tartronate. Moreover, dramatic increases were seen in some metabolites after only four hours of pre-analytical storage, such as lactate, pyruvate, fumarate, and most significantly, 5-oxoproline (See Figure 1.2.1) (Jain *et al.*, 2017). 5-oxoproline is an analyte of erythrocyte metabolism (Palekar, Tate and Meister, 1974). By contrast, other analytes are stable in serum after a prolonged storage of up to 24 hours (Breier *et al.*, 2014). In conclusion, due to the fragile characteristics of some metabolomes, it becomes essential to determine their stability limits. Furthermore, this ability to differentiate makes metabolomics a sensitive tool to test the sample quality.

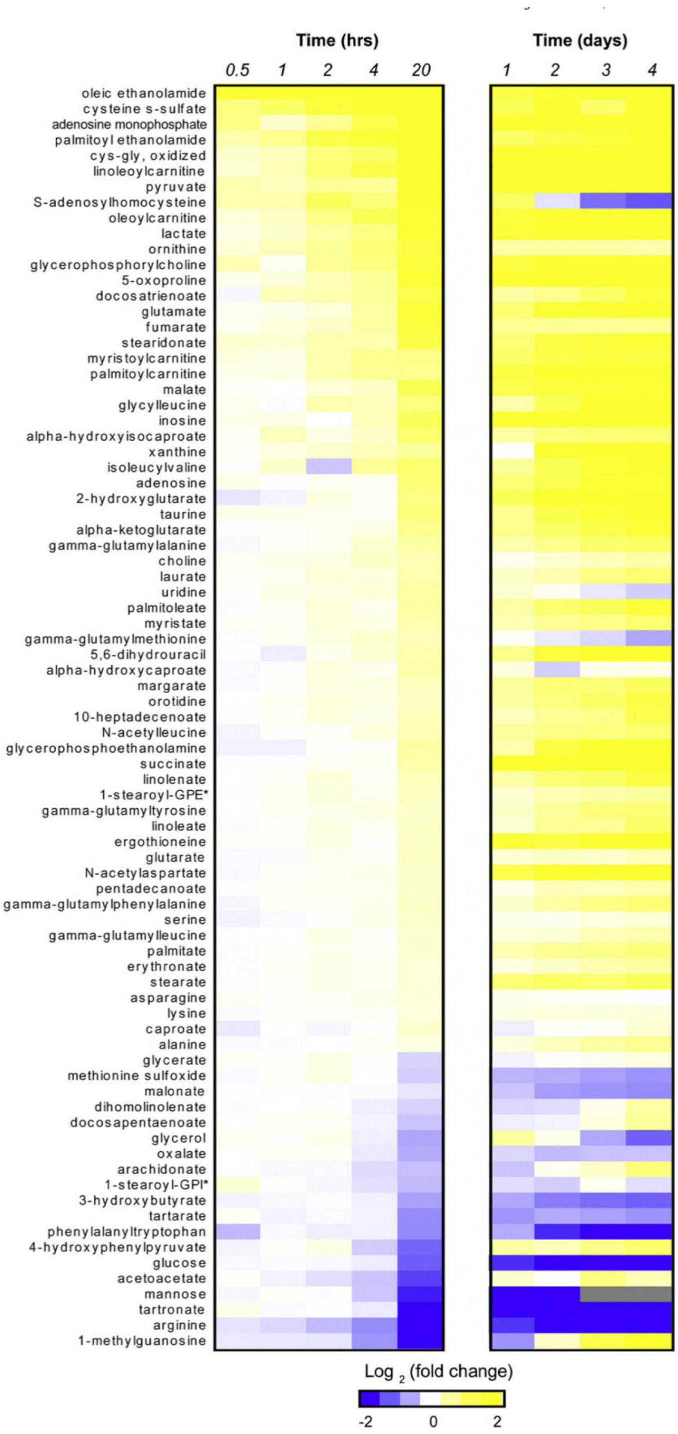

Figure 1: Metabolites disturbances during processing delays. The heat map demonstrates the fold change values across two independent periods, the first up to 20 hours and the second up to four days. for a group of metabolites *GPE = glycero-3-phosphoethanolamine; GPI= glycerophosphoinositol. Reprinted from Publication: Jain, M. et al. (2017) 'Analytes related to erythrocyte metabolism are reliable biomarkers for preanalytical error due to delayed plasma processing in metabolomics studies', Clinica Chimica Acta, 466, pp. 105–111. doi:10.1016/j.cca.2017.01.005. with permission from Elsevier license: 5305881417468**.**

1.4 Impact of other intrinsic and extrinsic factors on metabolomics

Noteworthy changes in metabolomics have been reported in various acute and chronic disorders, such as in insulin-resistance, type 2 diabetes mellitus (T2DM), cardiovascular disease (CVD) (Holland and Summers, 2008), atherosclerosis (Nelson *et al.*, 2006), neurodegenerative disorders (Piccinini *et al.*, 2010), alcohol-induced liver injury (Manna *et al.*, 2010), and various cancers (Cheng *et al.*, 2005; Chan *et al.*, 2009). Changes have also been reported in environmental factors, such as ionizing radiation in cellular and rodent models (Patterson *et al.*, 2008). This will also make metabolomics a sensitive tool to detect abnormal conditions and diseases insofar as the prognosis of conditions. Furthermore, the above studies reveal that by identifying an alteration in the metabolomics, scientists might successfully recognize the mechanisms of diseases and health disorders. This may enable them to potentially extrapolate the outcomes of the health conditions. Below, we will review the literature for more common factors which the present study is concerned with, as they may potentially impact the metabolomics quality.

1.4.1 Gender

Biological differences between females and males are well established, such as different levels of steroids, testosterone and oestrogen. Furthermore, various studies have demonstrated the differences in most of the major metabolism features between males and females. For example, the concentrations of amino acids in males such as lysophosphatidyl cholines, branched-chain amino acid, and creatinine are found to be higher in males than in females (Krumsiek *et al.*, 2015; Trabado *et al.*, 2017). On the other hand, oestrogen treatment is found to affect the regulation of Sphingomyelins (SMs) metabolism, and is found to be higher in females (Merrill *et al.*, 1985; Trabado *et al.*, 2017).

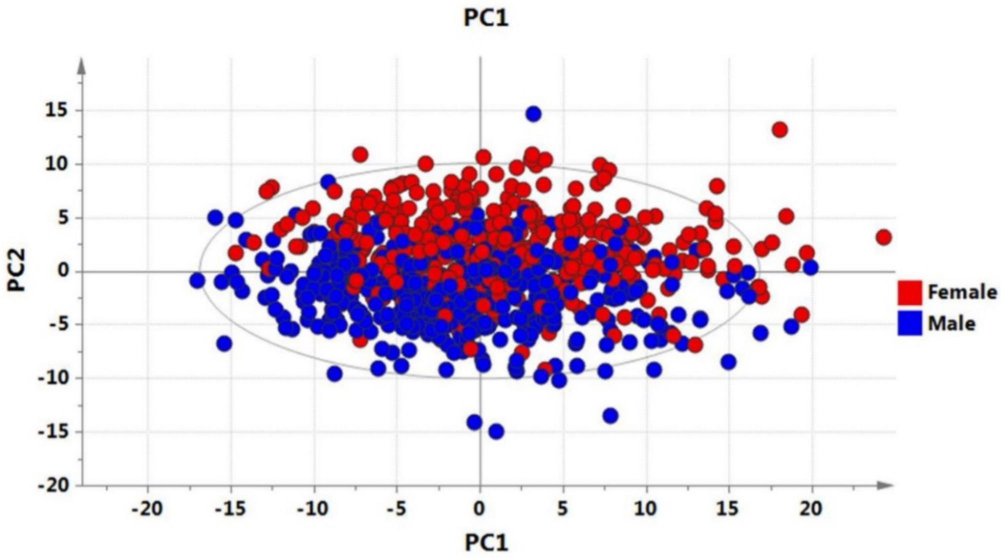

Figure 2: Principal components analysis (PCA) of metabolic profiles. PC1 (principal components 1) vs. PC2 (principal components 2) scores plot from PCA. Colours represent genders. Red dots, females, are largely represented on the superior side of the PC2, while blue dots, male, are largely represented on the inferior side of the PC2. Reproduced from (Trabado *et al.*, 2017) Fig 1, copyright CC BY 4.0.

1.4.2 Age

Age is known to affect various biological processes. Several studies have demonstrated significant changes in metabolomics with age progression. For example, a positive correlation was seen between the concentrations of total phosphatidylcholines and sphingomyelins and ageing. Additionally, Sphingomyelinase activity was reported to increase with age (Yu *et al.*, 2012; Mielke *et al.*, 2015; Trabado *et al.*, 2017). Other metabolites are reported to have different concentrations across age groups, such as amino acids (Chan, Suzuki and Yamamoto, 1999), succinate, isocitrate, malate, and lactate (Lawton *et al.*, 2008), and lipids (Lucio *et al.*, 2010). In addition, other studies by Vaarhost et al. (2011) and Gonzalez-Covarrubias et al. (2013) have connected higher levels of sphingomyelins to healthy ageing and longevity. This confirms age as a potential factor which may impact the metabolomics norm.

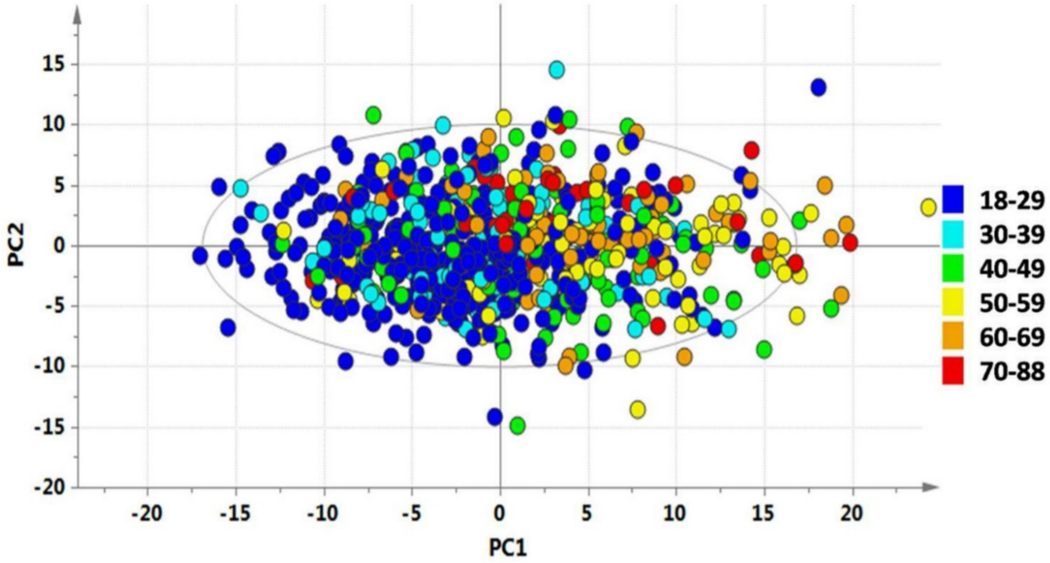

Figure 3: Principal components analysis (PCA) of metabolic profiles. Colours represent age groups. The aging tendency is seen along with PC1 (principal components 1). Reproduced from (Trabado *et al.*, 2017) Fig 1, copyright CC BY 4.0.

1.4.3 Body Mass Index

Obesity and high Body Mass Index (BMI) are linked to numerous health conditions, such as metabolic syndrome, T2DM, CVD and hypertension, as demonstrated by numerous studies (Newgard *et al.*, 2009; Yin, Lehmann and Xu, 2015; Trabado *et al.*, 2017). The studies have found body mass to impact metabolomics in various ways. For example, Newgard et al. (2009) have reported several changes in lipid homeostasis in participants with a BMI above 30 kg/m2. Several fatty acid species were also found to be significantly higher in the total fatty acid pool in these participants. Other studies have also found differences in steroid levels across measures of BMI (for example, Lucio et al. 2010). This reveals the importance of BMI as a potential factor to which attention should be paid for the study of metabolomics and their quality.

1.4.4 Diet and microbiome

The impact of dietary patterns on physical health in the short- and long term is well established. They have been found to affect potentially every aspect of human health and wellbeing (Schulze and Hu, 2002; Jannasch, Kröger and Schulze, 2017; Owen and Corfe, 2017). In comparison to an omnivorous diet, a vegan diet is found to have a lower risk of cardiometabolic disorders like T2DM and CVD (Benatar and Stewart, 2018). This impact of diet on health may reveal the potential presence of the effect of dietary patterns on various biological regularities. Studies have considered the direct and indirect influence of different diets on metabolomics (Wu *et al.*, 2016; Jin *et al.*, 2019). Specifically, a high consumption of red and processed meats, refined grain products and carbohydrate-rich dessert diets, such as the western diet, is associated with different levels of amino acids compared to diets rich in whole-grain products, vegetables, fruits and non-hydrogenated fat (Bouchard-Mercier, 2013). The authors have also reported that the western diet is associated with elevated levels of amino acids, including branched-chain amino acids and short-chain acylcarnitines. Furthermore, other metabolomes, such as catechol sulfate, hippurate, 3-hydroxyhippurate, 4-ethylphenylsulfate and 4-vinylphenol sulfate, are found to be significantly higher in the vegan diet than in omnivorous diets (Figure 4) (Wu *et al.*, 2016). In conclusion, the studies seem to indicate that people with different dietary patterns are expected to have variation in their metabolomics.

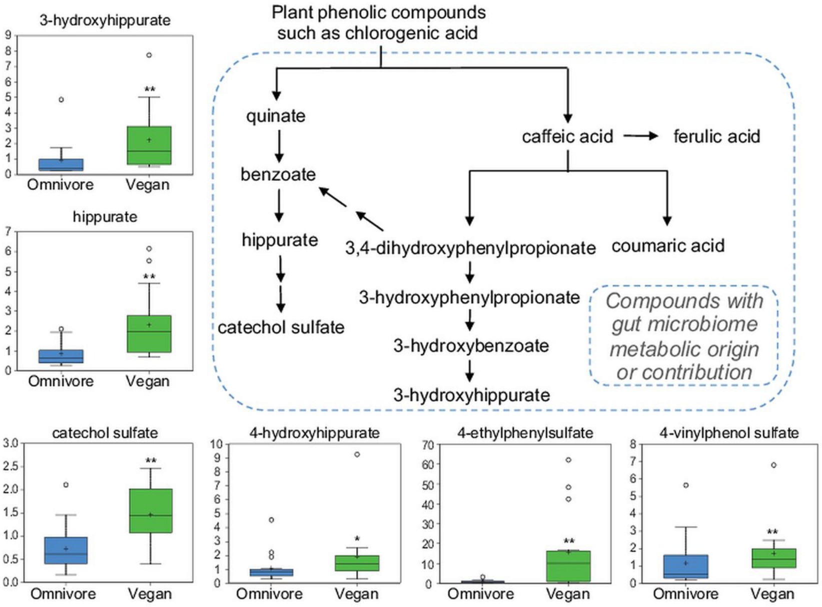

Figure 4: Plasma levels of metabolomes in people who follow vegan and omnivore diets, derived from gut microbiota metabolism. *p<0.1, **p<0.05. Reproduced from (Wu *et al.*, 2016) ‘Comparative metabolomics in vegans and omnivores reveal constraints on diet-dependent gut microbiota metabolite production’, *Gut*, 65(1), pp. 63–72. doi:10.1136/gutjnl-2014-308209. with permission from BMJ Publishing Group Ltd. license: 5306360586325

Moreover, the human metabolome and its microbiome can be interrupted through different factors, including diet and medication (Marcobal *et al.*, 2013; Wu *et al.*, 2016). Interestingly, the metabolic capabilities of microbiome are highly enhanced compared to the metabolic capabilities of the human body. The gut microbiome, for example, encodes around ten million genes (Li *et al.*, 2014)in comparison to the human body, which encodes around 19,000 genes (Theriot *et al.*, 2014). Studies observing microbiomes’ effect on mice and human metabolomes have found the gut microbiome to have a profound effect on many metabolites of the host. This effect has been detected in blood, in faeces and in urine (Wikoff *et al.*, 2009; Marcobal *et al.*, 2013). Disturbing the host-microbiome will lead to changes in the host-metabolomics. The administration of antibiotics, for example, has led to changes in many molecules and has affected the metabolism pathways in host-faeces. These changes have been found to include an elevation of non-consumable plant-polysaccharides (Marcobal *et al.*, 2013) and elevation of tryptophan transformation into tryptamine (Martin *et al.*, 2007; Wikoff *et al.*, 2009; Antunes *et al.*, 2011; Marcobal *et al.*, 2013), as well as an elevation of creatine and its derivative, creatinine (Romick-Rosendale *et al.*, 2009; Swann *et al.*, 2011; Marcobal *et al.*, 2013). In conclusion, the stability of the microbiome, and the factors which may interrupt it, such as lifestyle and medication, could lead to the alteration of metabolomics.

1.4.5 Overnight fasting

In common clinical practice it is routine for some blood analyses to be required after overnight fasting. Numerous studies have addressed the necessity of overnight fasting to achieve good quality of diverse molecules in blood samples (Kahleova *et al.*, 2017; Edinburgh *et al.*, 2018; Terada *et al.*, 2019). The impact of breakfast is not the same on all biological factors. However, studies have revealed that it would be sound practice to consider overnight fasting as an essential factor to exclude any potential impact of breakfast on blood sample quality (Legakis *et al.*, 2016; Longchamp *et al.*, 2017). Indeed, different types of breakfasts have been shown to have varied impacts on metabolomics. Foremost, as coffee and tea are commonly consumed as part of breakfast, coffee consumption with breakfast is reported to increase the concentration of some metabolomes, such as 5-hydroxymethyl-2-furoic acid and 2-furoylglycine, in urine. Furthermore, in comparing different types of breakfasts, egg and red meat, such as ham, has been found to increase the concentration of citrate and phosphocreatine/creatine ratio in urine, as compared to cereal (Rådjursöga M et al. 2017).

1.4.6 Circadian rhythm

A number of studies have discussed the strong relationship between circadian rhythm and metabolism (Ang *et al.*, 2012; Dallmann *et al.*, 2012; Bhadra *et al.*, 2017). With rhythm, organisms are able to attain the best of natural resources, preliminary sunlight, temperature and nutrition (Persson and Persson, 2018). The human body persists in a rhythm with the time zone according to the day-night cycle. Furthermore, travelling around the globe overtaking the earth's pace, mental status, sleep-wake cycle, and eating habits are temporarily disturbed with the change of time-zone, surpassing the biological clock toleration. Regardless, the body physiology is re-synchronised over time (Bhadra *et al.*, 2017). The circadian cycle can impact numerous metabolomics, either through direct impact, or indirect impact - as a result of associated food intake timing, for example. Ang JE et al. (2012) found around 19% of the identified metabolomes to be affected by the time of day. Moreover, Dallmann et al. (2012) found around 15% of the identified metabolomes in saliva and plasma to be under the direct control of the circadian rhythm. The metabolites found to be most affected by the circadian rhythm include the following: amino acids, lactate, carnitines and lipids (Ang *et al.*, 2012; Dallmann *et al.*, 2012). Therefore, in order to achieve sample quality, the impact of different times of day should be kept to a minimum. This was taken into consideration with the homogenous group in the present study by limiting the blood sampling to a narrow window of time.

1.4.7 Medications and supplements

Another factor which may impact the metabolome in various ways are medications and supplements. The impact can be direct, or indeed indirect, the latter through altering the microbiome or circadian rhythm, as discussed above. Examples of medication, including antibiotics and steroids, will be discussed below, as well as examples of supplements. A single dose of Dexamethasone, which is a commonly used short-term steroid, has been reported to strongly deregulate the metabolomes as well as the circadian rhythm (Bordag *et al.*, 2015). In addition, Cefoperazone, a broad-spectrum cephalosporin antibiotic, has been found in an in-vivo study to significantly alter the microbiome and gut metabolome (Gutierrez *et al.*, 2020). Finally, vitamin D supplements have been found to alter some oxidative stress-related metabolomes (Bhargava *et al.*, 2017; Raygan *et al.*, 2018). Taking medications and supplements into consideration, therefore becomes an essential part of the process in ensuring the sample quality of metabolomics.

The goals of the present study are to identify any metabolomics changes after preanalytical storage of the blood sample for three hours compared with blood samples after immediate centrifugation and to find if other intrinsic and extrinsic factors will affect short-storge impact on metabolomics stability. For this purpose, we recruited two groups of participants. The first group, the homogenous group with the minimum biological variation. The second group, the heterogeneous group, with at least one biological variation. We hypothesis first, short storage will impact the stability of the metabolomics. Second, intrinsic and extrinsic factors might affect the way in which short-storage impacts the metabolomics stability.

2 Study rationale

Metabolomics is a tool for detecting prognostic biomarkers of various biological changes and disorders. They may have the potential to achieve an early and accurate diagnosis and treatment. Understanding metabolomics is challenged by ensuring their quality and the factors that may impact their state. Moreover, in metabolomics and other analysis, a robust, user-friendly, practical, and economically viable measurement tool is indispensable for ensuring the blood sample quality. Most importantly, errors in the blood sampling stage potentially can lead to significant misleading laboratory results, including metabolomics. Whereas pre-analytical short storage errors are one the most common errors. Therefore, understanding these changes, as well a sufficient accurate, solid, and fast blood sample quality assessment method is highly warranted.

For this purpose, we would like to detect metabolomics changes after delayed storage using PESI-MS analysis technique in order to understand the short-storage impact as well as to evaluate the analysis technique for blood sample quality control. Understanding metabolomics and establishing a detection method for sample quality will help improve patients' health care, improve medical research, and reduce costs.

2.1 Study aims

The present study aims to predict metabolomics changes in blood samples after short storage compared with immediate centrifugation and to find if other intrinsic and extrinsic factors affect the concerned short-storge impact. Furthermore, to evaluate the PESI-MS as quality control of blood samples.

2.2 Hypothesis

First, we hypothesize that pre-analytical short storage will impact the metabolomics quality. second, that, intrinsic and extrinsic factors will affect the preanalytical short-storage impact. Third, PESI-MS is an appropriate technique for blood sample quality.

3 Method and Materials

The study was conducted by multidisciplinary team, where the author of this thesis took the position of applying the clinical part of the trial on volunteers, including explaining the trial, history taking, physical examination, blood sampling, volunteers health safety within the trial, data upload and ensuring applying the protocol, in addition to the academic part of this thesis, including the data analysis.
The study protocol have been published under :(Federal Ministry of Education and Research, 2014)

3.1 Participants and Design

50 volunteers were recruited to the study and divided into two groups. The first group, the homogenous group, consisted of a purposeful sample of 10 females and 10 males. This group was selected in order to reduce the metabolic variation.
The inclusion and exclusion criteria for participants are specified in table 2.

The second group, the heterogeneous group, consisted of 30 volunteers. This group of 14 females and 16 males was selected to reach broader metabolic variation with the aim of strengthening the trial sensitivity and to test the impact of the intrinsic and extrinsic factors on the short storage impact on metabolomics. The heterogeneous group participants were selected to include at least one of the intrinsic factors in table 3. The inclusion and exclusion criteria for participants are specified in table 2.

Table 2: Inclusion and exclusion criteria for the groups

Homogenous group	**Heterogeneous group**
Inclusion Criteria	
• Male or female	• Male, female or transgender
• Caucasian	• Any ethnicity
• Absence of existing health problems	• Preferably individuals with a pre-existing health condition or any condition which may impact the metabolic variation (Table 3)
• Age 20 to 30 years (Figure 5)	• Age 18 to 90 years (Figure 5)
• Body mass index (BMI) between 18.5 and 25 kg/m^2 (inclusive)	• BMI $\geq$ 18.5 kg/m^2
• Normal body temperature (within 35.9–37.6°C) on the day of blood sampling	
• Alcohol consumption $\leq$ 7 units/week (1 unit = 10 ml or 8 g alcohol)	
• Smoking /any other nicotine intake $\leq$1 cigarette/week or equivalent	
• Overnight fasting (12h)	
• Abstinence from physical exercise (24h)	
Exclusion criteria	
• Any special diet (for example, vegan, raw, vegetarian, intermittent fasting, ketogenic, no-carb, gluten free) • Any medication, or hormonal contraception, within the preceding 4 weeks • Drug abuse in the preceding 12 months pre-trial • In vitro fertilization (IVF) treatment in the preceding three months	

• Surgery in the preceding 3 months pre-trial	
• Severe physical health condition (chronic or acute) • Donation of blood or plasma in the preceding month, • Pre-existing anaemia • Gestation, breastfeeding, irregular or inadequate contraception, or intention of becoming pregnant • Language barriers • Mental incapacity	

Table 3: Intrinsic factors for the heterogeneous group

- Non-standard diet (for example, vegan, raw, vegetarian, Atkins low carbohydrate, intermittent fasting, Ketogenic)
- Non-Caucasian ethnicity
- Age > 30 years
- BMI > 25 kg/m2
- Chronic, non-severe metabolic disease (for example, diabetes, hypertension, obesity)
- Chronic, non-severe autoimmune disease (for example, rheumatoid diseases, systemic lupus erythema, multiple sclerosis)
- Chronic, non-severe lung disease (for example, pulmonary hypertension, asthma, chronic obstructive lung disease)
- Partial or total removal of organs (for example, bariatric surgery, hysterectomy, kidney donation) or transplant receiver
- History of substance abuse (for example, alcohol, drugs, smoking)
- Digestive disorder or malabsorption requiring a specific diet (for example, fructose-free, gluten-free, Crohn's disease, Phenylketonuria)
- Concomitant medication (for example, nonsteroidal anti-inflammatory drugs, heparin, corticosteroids, growth hormones, antihistamines or selective serotonin reuptake inhibitors)
- Menopausal females
- Acute, non-severe disease (for example, mild fever, common cold, recovering from a bone fracture or surgery)
- Any type of hormonal contraception (for example, oral, coil, implanted, 3-month injection)
- As judged by the investigator and accordingly noted in the source data form

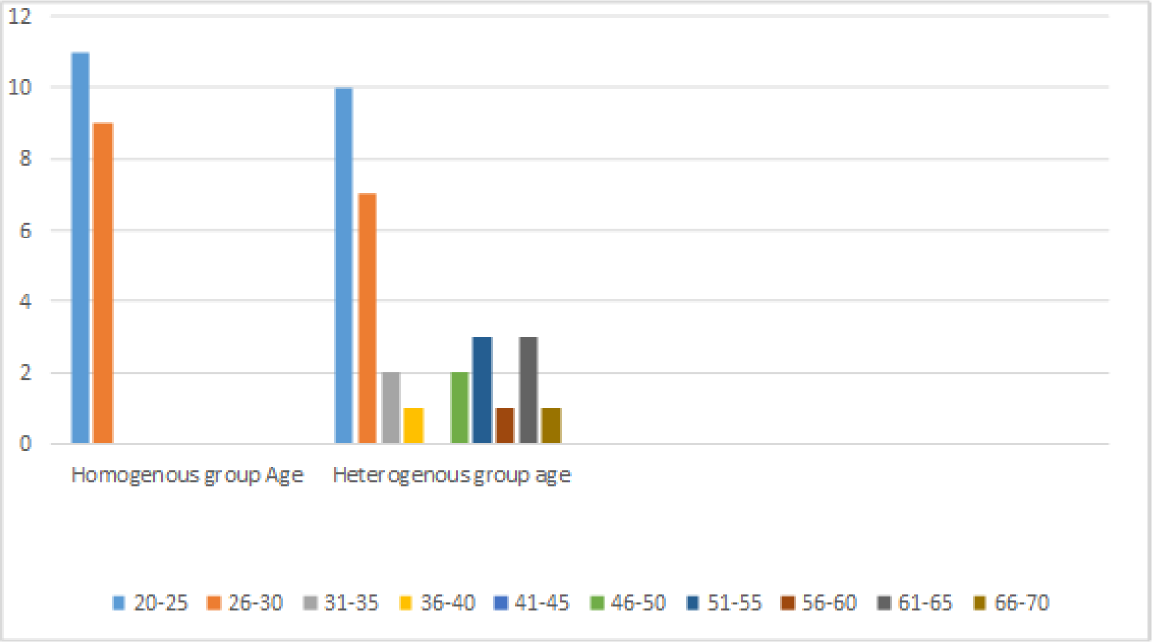

Figure 5: Age distribution of participants for both groups. Colours represent age groups.

3.2 Materials and Procedures

3.2.1 Blood sampling and plasma preparation

The collection of whole blood samples was initially conducted 8:00 am - 10:00 am for the homogenous group, and 8:00 am - 12:00 pm for the heterogeneous group. The following measures were applied to ensure sampling quality: 21G butterfly needle; the phlebotomy was performed only once per arm; the tourniquet was released within one minute; and the full sampling was completed within nine minutes.

EDTA-vacutubes were used throughout, and the tubes were gently overturned three times post-sampling. Dual samples, control samples and case samples, were taken in succession. The control samples were centrifuged immediately and the case samples were left upright at room temperature (logged, 21 to 26 °C) for three hours prior to centrifugation. Centrifugation was conducted for all samples at 2200 g at 4 °C for ten minutes before harvesting plasma. Plasma Aliquots were flash-frozen and transported on dry ice to be stored at -80°C until measurements were conducted. Measurements were then taken within two months of storage at -80°C.

While measurements by (PESI-MS) and data extraction and normalization were done by other team member (Bordag *et al.*, 2021).

3.2.2 Data analysis

For this work I analysed the data which were extracted and normalized by my supervisor Dr. Natalie Bordag. Sample measurements, data extraction and data transformation is detailed in our preprint (Bordag *et al.*, 2021). In short, 100 aliquots from the 50 volunteers were measured in duplicates, duplicates were averaged and a total of 4702 features were extracted. These feature were strictly filtered by their analytical quality (signal intensity, technical variability, missing data and background signal intensity) to retain 1200 features suitable for further statistical analysis. I performed further statistical analysis with MetaboAnalyst 5.0 (*MetaboAnalyst*). The missing values were estimated with KNN (feature-wise) and data was centred and scaled with Pareto scaling. Data analysis was carried out in two main parts. The first part consisted of identifying the impact of intrinsic factors upon metabolomics by testing for any significant differences between the two groups. The homogeneous and the heterogeneous groups were tested at zero hour and three hours independently. The second part was consisted of testing for any significant impact of short storage for three hours, as an extrinsic factor, upon the metabolomics. The homogenous and the heterogeneous groups were tested together to test for any significant differences between the control samples, which were centrifuged immediately, and the case samples which were centrifuged after three hours of short storage at room temperature. T-tests were applied to determine the existence of any significant differences between the groups. A multiple correction test was applied for false discovery rate (FDR) in order to achieve increased confidence in the significant data variation. Results are presented as boxplots, principal components analysis (PCA), and a heat map.

3.2.3 Research Funding

Part of this work has been carried out with the Competence Center CBmed, funded by the Austrian Federal Government within the COMET K1 Centre Program, Land Steiermark and Land Wien. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

4 Results

All volunteers reported no adverse event during or after donating their blood for this study. All blood samples showed no visible signs of possible other pre-analytical quality issues such as haemolysis. The measurement of 100 aliquots from the 50 volunteers (each at 0 h and 3 h) yielded 1200 PESI-MS features suitable for further statistical analysis. This constituted 574 features with positive-ion mode and 626 features with negative-ion mode., and. Below are the results of the three tests which were run in the study:

4.1 Homogenous verses heterogeneous after immediate centrifugation

Two groups were included in the trial to increase sensitivity by encompassing a broader metabolic variation. The homogenous and heterogeneous groups were included. The heterogeneous group was differentiated with the inclusion of at least one of the intrinsic factors which may impact the metabolomics (Table 3). Tests of significance were applied after immediate centrifugation. A t-test (with multiple correction test for false discovery rate FDR) was applied. No significant differences were found.

Principal components analysis (PCA) was used as a sample quality control (Figure 6).

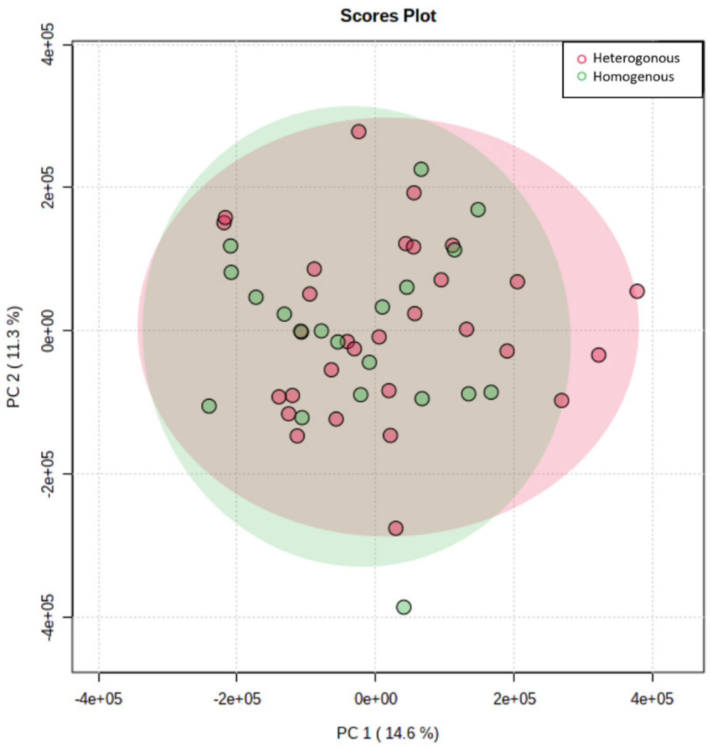

Figure 6: Principal components analysis (PCA): Circles represent groups. Each sample is represented by a dot. The closeness of the samples is a representation of the similarity. The figure shows no samples were seen as outliers.

4.2 Homogenous verses heterogonous group results at three hours

A t-test (with multiple correction test for false discovery rate FDR) was repeated for the samples of both groups after three hours of short storage at RT. No significant differences were found.

Principal components analysis (PCA) was used as a sample quality control (Figure 7).

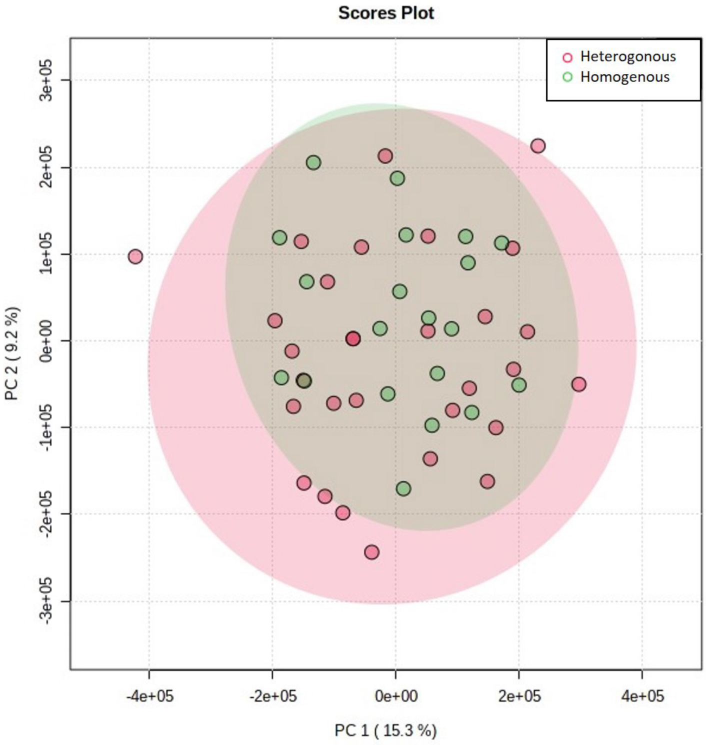

Figure 7: Principal components analysis (PCA): Circles represent the two groups, and each sample is represented by a dot. The closeness of the samples is a representation of the similarity. The figure shows no samples were seen as outliers.

4.3 Impact of short storage for three hours at room temperature.

The impact of short storage for three hours as an extrinsic factor on the metabolomics was tested at this stage. This was done by testing the two groups together for any significant differences between the control samples and case samples. Control samples were centrifuged immediately and case samples were centrifuged after 3 hours of short storage at RT. A t-test (with multiple correction test for false discovery rate FDR) was applied to identify any significant differences. Results revealed 10 highly significant variations (FDR < 0.05), and 1 significant variation with FDR: 0.05 (Table 4), (Figure 8-11).

Principal components analysis (PCA) was used as a sample quality control (Figure 10)

Table 4: Significant features identified by t-tests

	Features	t.stat	p.value	FDR
1	Neg. 88.99	14.6	$2 * 10^{-26}$	$2.97 * 10^{-23}$
2	Neg. 448.3	10.6	$7 * 10^{-18}$	$4.33 * 10^{-15}$
3	Neg. 446.75	10.1	$7 * 10^{-17}$	$3.04 * 10^{-14}$
4	Neg. 418.82	7.2	$1 * 10^{-10}$	$3.74 * 10^{-08}$
5	Neg. 420.37	6.5	$3 * 10^{-09}$	$7.74 * 10^{-07}$
6	Neg. 625.92	6.3	$9 * 10^{-09}$	$1.86 * 10^{-06}$
7	Neg. 419.6	4.4	$3 * 10^{-05}$	0.0056
8	Neg. 899.64	4.1	$7 * 10^{-05}$	0.011
9	Neg. 873.93	3.9	0.0002	0.021
10	Neg. 787.84	-3.7	0.0003	0.038
11	Neg. 710.21	3.6	0.0005	0.05

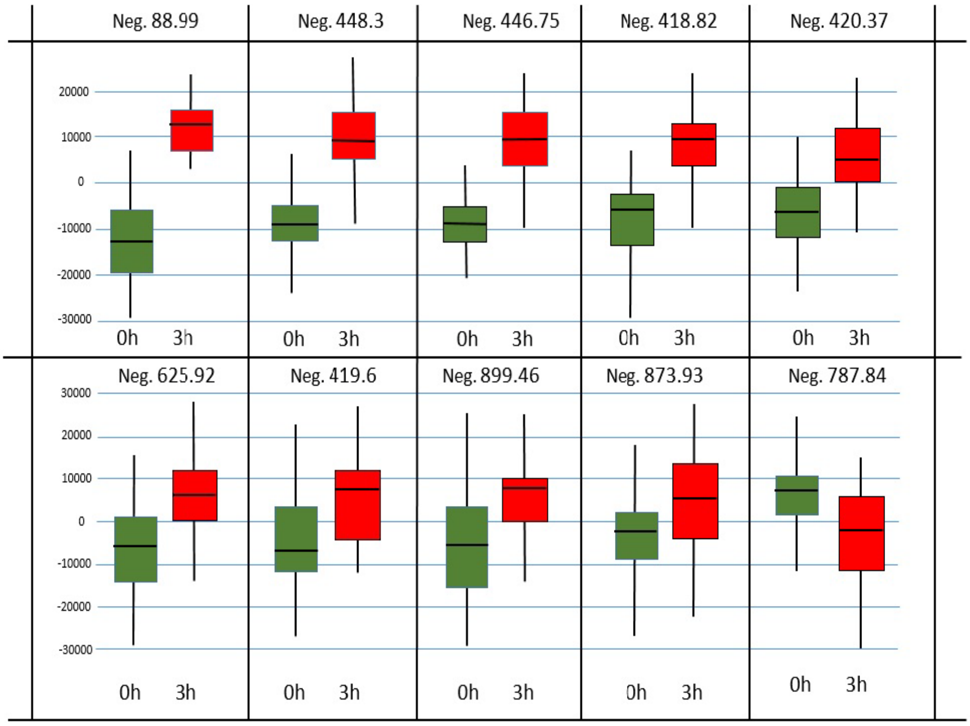

Figure 8: Box-plots of 10 changed features (highly significant) identified with t-test (before and after short-term storage for 3 hours at RT)

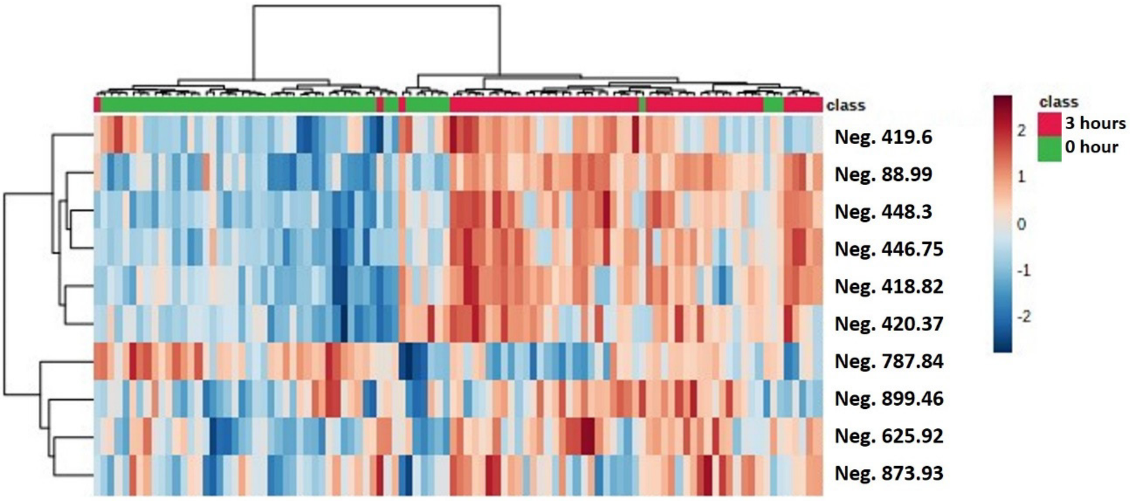

Figure 9: Heat map of the 10 features with highly significant differences before and after short-term storage for 3 hours at RT.

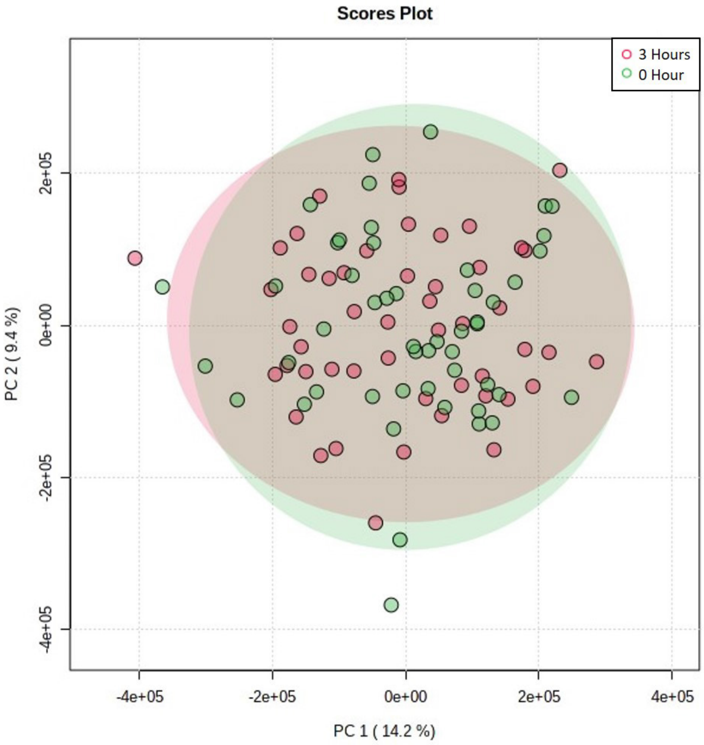

Figure 10: Scores plot between the selected PCs. The explained variances are shown in brackets. Circles represent groups and every sample represented by a dot and the nearness of the samples is a measurement of the similarity. The figure shows no samples were seen as outliers.

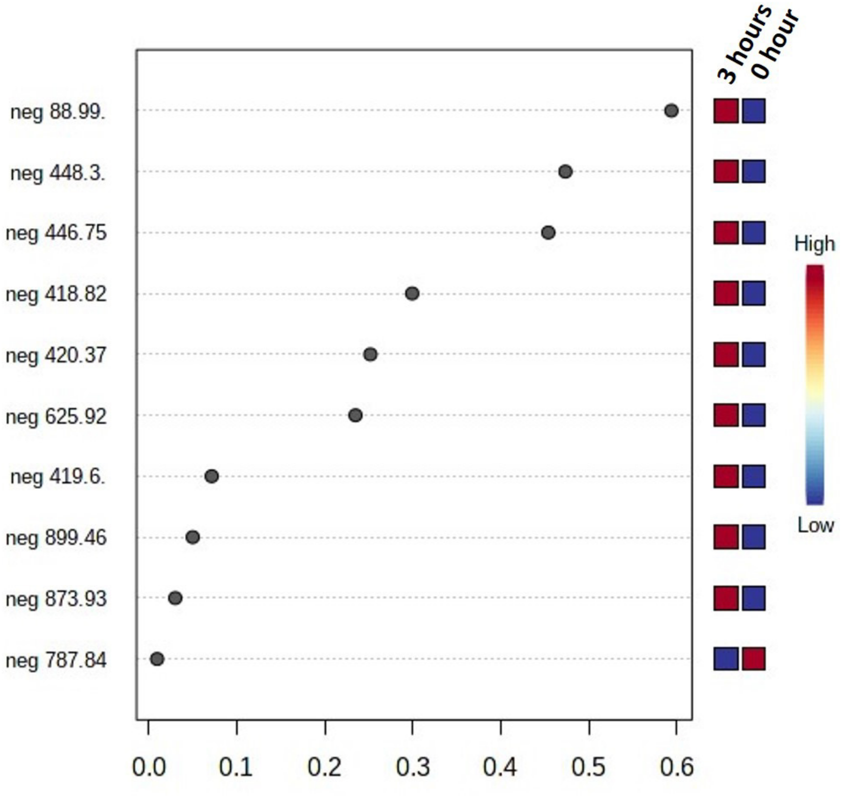

Figure 11: Partial least squares discriminant analysis (PLS-DA) of the 10 features with highly significant differences after short-storage at RT. The coloured boxes on the right indicate the relative concentrations of the corresponding metabolite in each group under study.

5 Discussion

This study investigated whether short-storage of blood samples for three hours at room temperature before centrifugation would impact metabolomics results. Our study also considered the potential effect of other intrinsic factors that may impact the outcomes. For example, age over 30 years, BMI over 25 kg/m2, restricted diet, smoking, or pre-existing health condition. Furthermore, we tried to understand which metabolomics components could be impacted. The study recruited two groups: a homogeneous group with the minimum metabolic variation and a heterogeneous group with at least one metabolic variation. The findings were that of the 1200 metabolomics results, the vast majority were stable after the short-storage period. The results are in line with existing studies (for example Yang *et al.*, 2013; Breier *et al.*, 2014). However, results also showed that around ten metabolites were found to be disturbed in the same period. This is in support of the first hypothesis. Such changes have also been identified in existing studies (Jain *et al.*, 2017). In contrast, other studies reported that around 22% of all metabolomics were significantly changed after a short storage of two hours at RT. One likely reason is the different measurement technology where the quantity and quality of the detected metabolomics are different (Kamlage *et al.*, 2014).

Some of the affected feature might be the result of glycolysis, like features neg. 88.99 and neg 418.82. The mass and ionization of feature neg. 88.99 correspond most to lactate. Lactate is the end product of anoxic glycolysis and erythrocytes are known to heavily rely on glycolysis as main energy source. Erythrocytes remain alive during the three-hour short/term storage at room temperature and thus remain metabolically active, using up glucose and increasing lactate levels, as also observed previously (Downes and Michell et al. 1981; Kamlage et al., 2014, 2018). Other affected features like neg. 873.93, and neg. 899.46 might be correspond to complex lipids (Heins, Heil, and Withold et al. 1995).

There were no differences observed between the homogeneous and heterogeneous groups. This finding does not support the second hypothesis which predicted that there would be a significant difference between the groups. These findings can lead us to conclude that short-storage for three hours could potentially impact sample quality. Moreover, the affected metabolomics (which might be erythrocytes related metabolomics) could be used as a detection method or tool to evaluate the sample quality.

Our findings offer a robust standpoint on PESI-MS sensitivity concerning the pre-analysis short-storage and potentially affecting metabolomics. Furthermore, our findings have clarified the question of potential effects of intrinsic and extrinsic factors on the short-storage impact. Specifically, our results showed no significant differences between the heterogeneous and homogeneous groups. Taken together, our findings point towards a potential value in using PESI-MS in evaluating blood samples quality.

Concerning the differences between the homogenous and heterogeneous groups and the impact of the concerned differences, contrary to other studies, our trial revealed no significant difference between the two groups. One possible reason for this could be the unfocused and relatively small sample size for every factor. Another reason could be the very different technology, as PESI-MS was used here, which is a new ionization technique with relatively lower quality and only single Q mass while MS is a high-resolution mass technique (Bordag *et al.*, 2021). However, PESI-MS offer a rapid, practical and economically viable measurement tool (Hiraoka *et al.*, 2020), opposite to MS which demand a highly specialized expertise in this technique. In addition, the procedures needed to achieve successful, high-quality research are expensive and complicated (Segers *et al.*, 2019). Finally, the risk of haemolysis, which may be a concern in blood sampling, was decreased by following a strict procedure: tourniquet release within one-minute, adequate needle size, and gentle sample shaking.

Limitations of the present study include the unfocused group of participants with different biological variables, and this may limit the likelihood of seeing a significant difference caused by these factors. As mentioned above, and not to study the particular impact of these biological variables. Other limitation can be the sample size, as 50 participants. may not represent the variation which seen in real practice, future studies should expand on wider range of participants.

Data quality was another limitation, as PESI-MS is relatively lower quality and only single Q, in comparison with MS. However, PESI-MS offer a rapid, practical and economically viable measurement tool (Hiraoka *et al.*, 2020).

Summary and conclusion

In this study, we investigated the impact of pre-analysis short-storage for three hours at room temperature on metabolomics with regard to intrinsic and extrinsic factors. The result showed that out of 1200 metabolomics results, the vast majority were stable after the short-storage period, which is supported by existing studies (Yang *et al.*, 2013; Breier *et al.*, 2014). And only a small minority of around ten metabolomics were found to be disturbed in the same period. Such changes have also been found in other studies (for example, Jain *et al.*, 2017). In addition, no discernible impact for the intrinsic and extrinsic factors on the metabolomics was observed after the short storage. Taken together, this offers a robust standpoint on the PESI-MS technique sensitivity with regard to the pre-analysis short-storage impact. Taken together, our results and the findings of earlier studies point towards a potential value in using the PESI-MS technique in blood samples quality control. Future research may extend this work by focusing on specific changes in metabolomics with regard to any potential corresponding health conditions.

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