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Metabolomic study of carotid–femoral pulse-wave velocity in women

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Objective: Carotid–femoral pulse-wave velocity (PWV) is a measure of aortic stiffness that is strongly associated with increased risk of cardiovascular morbidity and mortality. The aim of the current study was to identify the molecular markers and the pathways involved in differences in PWV in women, in order to further understand the regulation of arterial stiffening.

Methods: A total of 280 known metabolites were measured in 1797 female twins (age range: 18–84 years) not on any antihypertensive medication. Metabolites associated with PWV (after adjustment for age, BMI, metabolite batch, and family relatedness) were entered into a backward linear regression. Transcriptomic analyses were further performed on the top compounds identified.

Results: Twelve metabolites were associated with PWV ($P < 1.8 \times 10^{-4}$). One of the most strongly associated metabolites was uridine, which was not associated with blood pressure (BP) and traditional risk factors but correlated significantly with the gene-expression levels of the purinergic receptor P2RY2 (Beta = −0.010, SE = 0.003, $P = 0.007$), suggesting that it may play a role in regulating endothelial nitric oxide synthase phosphorylation. On the other hand, phenylacetylglutamine was strongly associated with both PWV and BP.

Conclusion: Circulating levels of uridine, phenylacetylglutamine, and serine appear strongly correlated with PWV in women.

Keywords: biomarkers, metabolomics, pulse-wave velocity

Abbreviations: BP, blood pressure; CIDEC, cell death activator CIDE; CSTL1, cystatin-like 1; eNOS, endothelial nitric oxide synthase; MAP, mean arterial pressure; PWV, pulse-wave velocity; SE, standard error; UTP, uridine triphosphate

INTRODUCTION

Carotid–femoral pulse-wave velocity (PWV), a measure of large artery stiffness, is a well known independent predictor of cardiovascular morbidity and mortality [1–4], and it is considered an integrative measure of the impact of cardiovascular risk factors [5]. Though arterial stiffening has been associated with ageing [6], hypertension [7], diabetes mellitus [8], hypercholesterolaemia [9,10], and chronic kidney diseases [11], its physiopathology is still not fully understood.

Recent advances in metabolomics have allowed for high-throughput assay of an extensive set of small molecules in a number of biological fluids. Low-molecular weight metabolites represent the intermediates and end-products of metabolic pathways that reflect physiological functions and, thus, may mirror the early stages of a pathological state [12].

A small study followed longitudinally 174 individuals and found that plasma fatty acid composition (assessing levels of 10 fatty acids) correlates with both PWV and mortality [13]. Full metabolomic profiling regarding PWV has not yet been attempted.

In this study, we performed metabolomic screening in a large cohort of women from TwinsUK to identify the novel metabolites that associate with PWV. We further explored the relationship between metabolites associated with PWV and gene-expression data to further understand the molecular mechanisms underlying arterial stiffening. We also explored the association of the identified metabolites with the Framingham 10-year cardiovascular risk assessment [14].

MATERIALS AND METHODS

Study population

The study participants were twins enrolled in the TwinsUK Registry, a national register of adult twins recruited as

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volunteers without selecting for any particular disease or trait [15]. All recruited twins were of the same sex. In this study, we analysed data from 1797 female twins who had complete data for body composition and metabolomics profiling.

The study was approved by the St. Thomas' Hospital Research Ethics Committee, and all twins provided informed written consent.

Pulse-wave velocity measurement

Carotid–femoral PWV was calculated from the sequential recordings of carotid and femoral artery pressure waveforms using the same SphygmoCor device and applanation tonometry. Difference in the time of pulse arrival from the R-wave of the electrocardiogram between the two sites was taken as the transit time, and the difference in path length was estimated using surface measurements with a flexible tape measure between the sternal notch and the point of applanation at the femoral artery as previously described. PWV is determined by dividing the distance by transit time. Coefficient of variation between operators was less than 10% [5]. Measurements were made in triplicate, and mean values were used for analysis. PWV measurements were available in 1797 women.

Metabolomics measurements

Nontargeted metabolite detection and quantification was conducted by the metabolomics provider Metabolon, Inc. (Durham, North Carolina, USA) on fasting blood samples, as described previously [16]. In this study, we analysed 280 structurally named biochemicals (known metabolites) categorized into the following broad categories – amino acids, acylcarnitines, lysophospholipids, carbohydrates, vitamins, lipids, nucleotides, peptides, xenobiotics, and steroids.

Muther expression data

The Muther gene-expression dataset consists of 825 abdominal fat samples. Gene expression was analysed with the Illumina Human HT-12 V3 (Illumina Inc., San Diego, California, USA) [17], 586 individuals entered the metabolite association analysis.

Statistical analysis

Statistical analysis was carried out using Stata version 11 (Stata Corp., College Station, Texas, USA). We inverse normalized the metabolite data, as the metabolite concentrations were not normally distributed. To avoid spurious false-positive associations because of small sample size, we excluded metabolic traits with more than 20% missing values. We imputed the missing values using the minimum run day measures.

We looked for the metabolites associated with PWV by running random intercept linear regression adjusting for age, BMI, metabolite batch, and family relatedness. We corrected for multiple comparisons using Bonferroni correction, thus giving a significant threshold of $P = 1.8 \times 10^{-4}$ (0.05/280 metabolites). We then used a stepwise backward regression model to identify a set of metabolites that were significantly associated with each phenotype using P less than 0.01 as cut-off threshold. As metabolites in their nature can be affected by many factors, in particular dietary factors [18], we run random intercept linear regressions to test the effect of fruit and vegetable intake and alcohol intake on the association between the metabolites and PWV.

Associations of metabolites with gene-expression levels in fat were tested using random intercept linear regression after adjusting for age, BMI, metabolite batch, expression batch, and family relatedness.

Finally, we explored the association of selected metabolites with Framingham 10-year cardiovascular risk [14] by running random intercept logistic regression adjusting for age, BMI, metabolite batch, and family relatedness.

RESULTS

The descriptive characteristics of the study participants are shown in Table 1. After adjustment for covariates, we identified 12 metabolites, whose levels significantly correlated with PWV (Table 2). We then proceeded to analyse, in a multivariate model, which of these metabolites contributed independently and identified only three metabolites: phenylacetylglutamine, serine, and uridine (Table 3). The proportion of the variance explained by the circulating levels of these three compounds is $R^2 = 30\%$. Of the three metabolites, only phenylacetylglutamine was associated with both SBP and DBP [SBP: −1.6 (−2.36; −0.84), $P = 4.1 \times 10^{-5}$; DBP: −0.88 (−1.33; −0.44), $P = 8.8 \times 10^{-5}$]; 10^{-5}]; however, the metabolite–PWV association remained significant even after adjusting for mean arterial pressure (MAP) in the linear model and after adjusting for dietary factors (fruit and vegetable intake and alcohol intake). Published studies have shown that PWV can predict cardiovascular risk that is not accounted by the traditional factors included in the Framingham risk score [19]. We therefore proceeded to assess whether these three metabolites were associated with the Framingham risk score. We find that phenylacetylglutamine is associated with the Framingham cardiovascular risk score [Beta = −0.04, standard error (SE) = 0.01, $P = 0.004$]. However, there was no association with both uridine and serine and the Framingham cardiovascular risk score (uridine: Beta = −0.003, SE = 0.01, $P = 0.8$; serine: Beta = −0.002, SE = 0.01, $P = 0.85$). Also, none of the three metabolites were associated with either total or HDL cholesterol. This suggests that some of the molecular pathways contributing to PWV are independent of the traditional cardiovascular disease risk factors measured by the Framingham score (Fig. 1).

TABLE 1. Demographic characteristics of the study population

Phenotype	TwinsUK
n	1797
Male: female	0 : 1797
Monozygotic twin: dizygotic twin:singletons	812 : 860 : 125
Age (years)	57.93 (9.17)
BMI (kg/m^2)	26.33 (4.80)
DBP (mmHg)	78.39 (9.48)
PWV (m/s)	9.33 (1.95)
SBP (mmHg)	127.03 (16.13)

Values are given as mean (SD). PWV, pulse-wave velocity.

TABLE 2. List of metabolites significantly associated with pulse-wave velocity after adjusting for age, BMI, experimental batch, family relatedness, and multiple testing

Metabolite	Super-p	Sub-p	Beta (95% CI)	*P*
Methionine	a-a	Cysteine, methionine, SAM, taurine metabolism	−0.24 (−0.34; −0.15)	1.33×10^{-6}
Glutamine	a-a	Glutamate metabolism	−0.22 (−0.32; −0.11)	6.47×10^{-5}
Glycine	a-a	Glycine, serine and threonine metabolism	−0.23 (−0.33; −0.14)	2.62×10^{-6}
Serine	a-a	Glycine, serine and threonine metabolism	−0.26 (−0.36; −0.16)	1.86×10^{-7}
3-Phenylpropionate (hydrocinnamate)	a-a	Phenylalanine and tyrosine metabolism	−0.18 (−0.27; −0.09)	6.69×10^{-5}
Phenylacetylglutamine	a-a	Phenylalanine and tyrosine metabolism	−0.17 (−0.26; −0.08)	1.37×10^{-4}
Indolepropionate	a-a	Tryptophan metabolism	−0.18 (−0.27; −0.09)	1.64×10^{-4}
Trans-4-hydroxyproline	a-a	Urea cycle; arginine and proline metabolism	−0.19 (−0.28; −0.1)	6.66×10^{-5}
Urea	a-a	Urea cycle; arginine and proline metabolism	−0.21 (−0.31; −0.11)	8.47×10^{-5}
Glycerate	ch	Glycolysis, gluconeogenesis, pyruvate metabolism	−0.19 (−0.29; −0.09)	1.28×10^{-4}
Threonate	c and v	Ascorbate and aldarate metabolism	−0.26 (−0.35; −0.16)	1.17×10^{-7}
Uridine	n	Pyrimidine metabolism, uracil containing	−0.26 (−0.34; −0.17)	9.03×10^{-9}

a-a, amino-acid; c and v, cofactor and vitamin; ch, carbohydrate; CI, confidence interval; n, nucleotide; sub-p, sub pathway; super-p, super pathway.

Uridine

Uridine is associated with lower PWV in our data (Table 2). When we tested for correlations between uridine and gene expression, we found that none of the expression probes passed Bonferroni correction for uridine. However, circulating uridine levels were nominally associated with the gene-expression levels in fat of P2RY2 (Beta = −0.010, SE = 0.003, $P = 0.007$), suggesting that it may play a role in regulating endothelial nitric oxide synthase (eNOS) phosphorylation which may in turn influence arterial stiffness. This association may be mediated through an effect on endothelial function independent of the classical risk factors. Uridine is known to be an agonist of P2 receptors, particularly the P2Y subclass which consists of eight known human P2Y receptors (1, 2, 4, 6, and 11–14). This is relevant as P2Y1, P2Y2, and possibly P2Y4 are the purinergic receptors involved in eNOS phosphorylation during endothelial activation [20].

Uridine is a nucleotide base used as a dietary supplement for increasing the synthesis of cellular membranes and for other neurological properties. Uridine is able to exert an acute cardioprotective effect against myocardial ischaemia when preloaded, which is abolished by blocking potassium channels on the mitochondria (with 5-hydroxydecanoate); it appears that uridine preloading preserves the levels of energy metabolites (ATP, creatine phosphate, and uridine) and subsequently reduced lipid peroxidation [21]. The nominal association with expression levels of a purinergic receptor suggests that uridine may exert its role on PWV via endothelial dysfunction [22].

Serine

Serine is one of the naturally occurring amino acids and it is synthesized in the body from other metabolites. It participates in the biosynthesis of purines and pyrimidines, and is also the precursor to numerous other metabolites, including sphingolipids and folate, the principal donor of one-carbon fragments in biosynthesis. In our data, we find that circulating levels of serine are significantly associated with lower PWV and with expression levels of a probe on the cystatin-like 1 (*CSTL1)* gene on chromosome 20 (Beta = 0.09, SE = 0.02, $P = 4.26 \times 10^{-8}$). The cystatin locus on chromosome 20 contains the majority of the type 2 cystatin genes and pseudogenes, and has been associated with cerebral haemorrhage and cerebritis [23].

Phenylacetylglutamine

Phenylacetylglutamine is a major nitrogenous metabolite that accumulates in uraemia [24]. It is the glutamine conjugate of phenylacetic acid produced in humans and is also a well known gut microbial cometabolite whose levels are significantly different between Asian and North American individuals [25]. Although Holmes *et al.* [25] did not measure the correlation between phenylacetylglutamine and blood pressure (BP), they did test that between BP and hippurate (another microbial cometabolite significantly different between Asians and Caucasians, positively correlated with phenylacetylglutamine) and reported a negative correlation with DBP. Such published results are consistent with our findings, that is, a negative correlation between phenylacetylglutamine and SBP, cardiovascular risk and PWV. In our data, we also find a weak negative correlation between hippurate and SBP (Beta = −0.97, SE = 0.39, $P < 0.012$).

Phenylacetylglutamine levels in our data are correlated with adipocyte gene-expression levels of the cell death activator CIDE (*CIDEC:* Beta = 0.05, SE = 0.01, $P = 6.97 \times 10^{-8}$). This gene is regulated by insulin and its expression is positively correlated with insulin sensitivity [26]. Mutations in this gene may contribute to insulin-resistant diabetes [27]. CIDEC plays an important role in energy

TABLE 3. List of metabolites significantly associated with pulse-wave velocity in the stepwise backward regression

Metabolite	Super-p	Sub-p	Beta (95% CI)	*P*
Phenylacetylglutamine	a-a	Phenylalanine and tyrosine metabolism	−0.13 (−0.22–0.05)	2.20×10^{-3}
Serine	a-a	Glycine, serine and threonine metabolism	−0.17 (−0.28–0.07)	1.20×10^{-3}
Uridine	n	Pyrimidine metabolism, uracil containing	−0.18 (−0.28–0.09)	1.00×10^{-4}

a-a, amino-acid; CI, confidence interval; n, nucleotide; sub-p, sub pathway; super-p, super pathway.

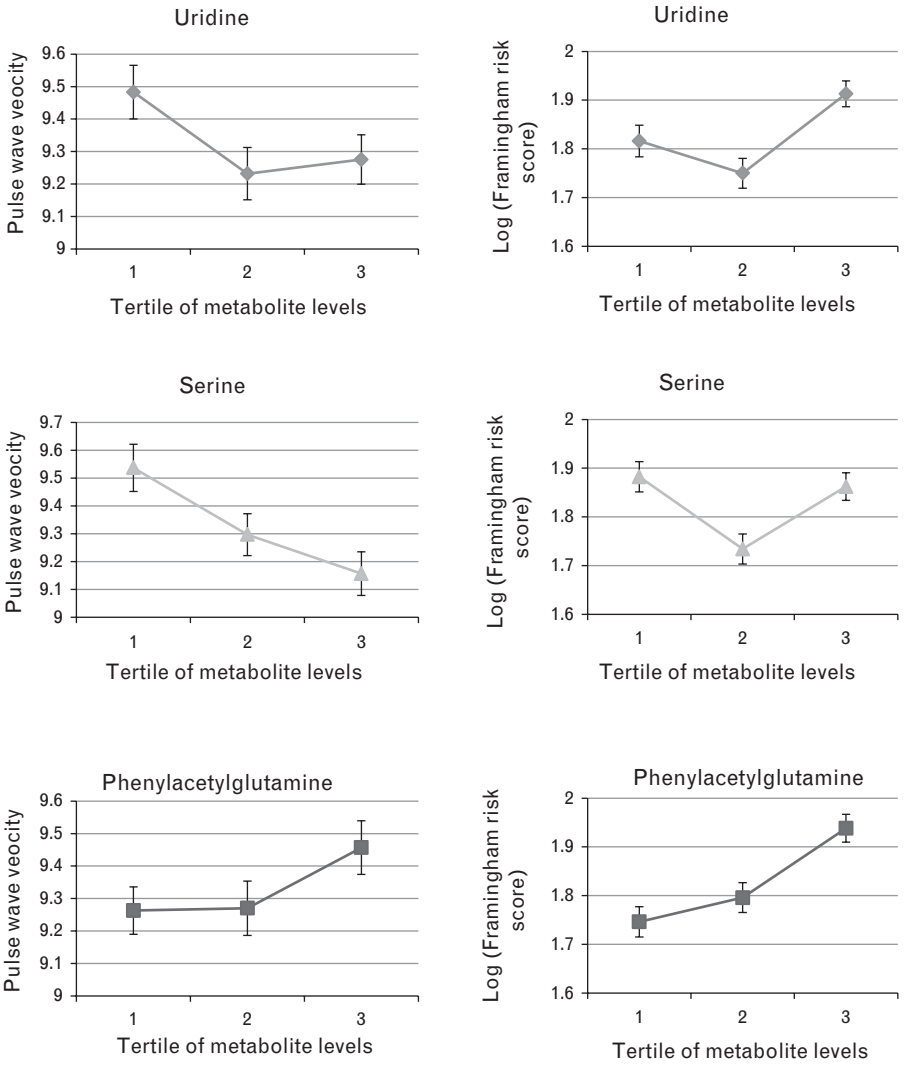

FIGURE 1 Metabolite associations with PWV and cardiovascular risk as measured by the Framingham risk scores. Mean PWV and log Framingham risk scores (adjusted for age, BMI and batch) are shown by tertiles of selected metabolites. PWV, pulse-wave velocity.

metabolism and lipid droplet formation [28], and its hepatic expression is increased in obese humans and is downregulated by marked weight loss [29].

DISCUSSION

Using metabolomic profiling, we searched for the molecular markers and the mechanisms involved in differences in PWV in women in order to investigate the regulation of arterial stiffening. We identified 12 blood metabolites, mainly amino acids, with high statistical significance associated with PWV. We also report three metabolites amongst those identified to be independently associated with PWV: uridine, serine, and phenylacetylglutamine achieving an R^2 of 30%. Of the three metabolites identified, the one showing the strongest association is uridine. Uridine triphosphate (UTP, which unfortunately is not measured by the current metabolomic panel) stimulates vasodilatation, automaticity in ventricular myocytes, and release of tissue-plasminogen activator, indicating that UTP may be important in cardiac regulation [30]. Uridine levels may be reflecting lower UTP levels or they may be cardioprotective via some other mechanism. Interestingly, however, uridine levels are not correlated with Framingham risk. We also report that circulating levels of serine are associated with PWV but not with Framingham risk. Our data indicate that the mechanisms underlying the association of PWV with uridine and serine are likely to be independent of the traditional CVD risk factors. Recent studies have shown that PWV improves cardiovascular event prediction [31,32]. Our data are, therefore, consistent and suggest that there are molecular mechanisms related to arterial stiffening and cardiovascular mortality that are not fully encompassed by the traditional cardiovascular risk factors.

We also report a novel association between phenylacetylglutamine and both Framingham risk scores and PWV. The negative correlation with phenylacetylglutamine is consistent with the previous reports on gut-microbiome-derived metabolites [25] and BP. We find that this metabolite is strongly associated with the gene-expression levels of *CIDEC*, a gene related to insulin resistance, suggesting that this metabolite may be related to this pathway.

Phenylacetylglutamine is related to the gut microbiome composition and a number of reports have linked the function of gut bacteria to insulin resistance. Therefore, it is possible that *CIDEC* may be linking these two pathways and suggests a new mechanism linking insulin resistance and gut microbiome in BP regulation.

Our study, therefore, on one hand is consistent with the current knowledge of insulin resistance and endothelial activation mechanisms in determining arterial stiffening. On the other hand, our data suggest that some of these mechanisms may be related to the mechanisms that deserve further exploration, for example, those that link with the gut microbiome. These data also suggest that serine and uridine levels are linked to arterial stiffening, possibly via endothelial dysfunction but in a way that is not reflected directly on the traditional CVD risk factors.

The current study has several strengths. It used a nontargeted metabolomic approach that identifies a wide range of biochemicals in addition to lipids. TwinsUK is a very large and accurately phenotyped population, and this allowed us to explore the potential confounders (e.g. diet and MAP). The availability of expression and genetic data enabled us to explore some of the biological implications of the three metabolites identified.

We note some study limitations. Our study sample consisted of women only, and some metabolites could be influenced by sex-specific hormones. In addition, previous studies suggested that traditional risk factors [33] are less reliable in predicting risk in women than in men. We have only tested individuals of European descent, and the levels of one of the metabolites identified (phenylacetylglutamine) are known to vary between Asians and Caucasians. More importantly, because of the novelty of the phenotypes, we could not validate our results in an independent cohort. The cross-sectional nature of our data does not allow us to draw conclusions as to whether the metabolites identified are causative of arterial stiffness or merely correlated with it. However, our results highlight the relevance of investigating the molecular pathways related to PWV as this may lead to the identification of molecular mechanisms involved in cardiovascular diseases, in particular linked to endothelial activation, that act through other pathways. The identification of key metabolites related to PWV should encourage further research into this field.

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Conflicts of interest

R.P.M. is an employee of Metabolon, Inc.

M.J.B. is a full-time employee of Pfizer; J.T. and M.J.B. are share holders of Pfizer.

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Reviewer's Summary Evaluation

Reviewer 1

This original study proposed by Menni *et al.* points to molecular pathways and biological processes involved in arterial stiffness in women. The identification of biological mechanisms affecting arterial viscoelastic properties paves the way for eventual specific treatment.