Lipidomic and metabolomic analyses reveal potential plasma biomarkers of early atheromatous plaque formation in hamsters

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**Aims**

Atherosclerosis is the main pathological process contributing to cardiovascular disease, with diet being the most important factor involved. Although the lipidome of atheromatous plaque has been studied previously, the use of comparative lipidomics and metabolomics in plasma in early atherogenesis could lead to the discovery of plasma biomarkers that allow not only disease prediction but also measurement of disease progression.

**Methods and results**

High-throughput techniques, such as liquid chromatography/mass spectrometry, allowed us to compare the circulating and aortic lipidome and plasma metabolome in order to look for new molecular targets involved in atherogenesis. To achieve this objective, we chose the hamster (Mesocricetus auratus) as the best small animal model for diet-induced early atherosclerosis, because its lipoprotein metabolism is similar to that of humans. The results revealed the existence of several, previously unreported, changes in lipid and amino-acid metabolism, the peroxisome proliferator-activated receptor \(\gamma\) pathway, and oxidative and endoplasmic reticulum stress, also involving cell senescence. Furthermore, as a proof of concept in the modelling of dietary influences in atherogenesis, we have measured the effect of a potential anti-atherogenic polyphenol extract on the reported pathways. Our results support a previously unknown role for taurocholic acid as a potential plasma biomarker of early atheromatous plaque formation.

**Conclusion**

The use of comparative liquid chromatography/mass spectrometry-based lipidomics and metabolomics allows the discovery of novel pathways in atherogenesis, as well as new potential plasma biomarkers, which could allow us to predict disease in its early stages and measure its progression.

**Keywords**

Sphingolipids • Free cholesterol • Taurocholic acid • Cell senescence • Phospholipid oxidation

1. Introduction

Atherogenesis, a major contributor to cardiovascular diseases (CVDs), is a pathological process with a multifactorial nature. Its diverse causal factors include age, sex, inflammation, oxidative stress, dyslipidaemia, changes in vascular flux, and diabetes, among many others.\(^1\) Despite this knowledge of general factors, one of the major challenges remains the assessment of the risk factors for atherogenesis at a clinical level, besides those validated epidemiologically, such as low-density lipoprotein (LDL)-cholesterol, lack of healthy exercise, dietary factors, and others.\(^1,2\) Although in situ morphological tests, such as imaging of vascular walls at selected locations, have a great potential, they are not easily undertaken in large population studies. Furthermore, most of these measurements detect only established plaques, so their usefulness in preventive approaches is still limited. It is therefore desirable to establish circulating biomarkers for the formation and progression of atheroma at earlier stages. As a local vascular tissue-based event, the markers of its progression from normal vascular cell homeostasis to plaque development and potential disruption need to be distinguished from normal variations of
potential circulating biomarkers related to specific risk factors. In this context, experimental models that reproduce the pathogenic steps could pave the way for the discovery of such biomarkers. Thus, the use of experimental models has facilitated the study of the disease, despite there being some controversy regarding the validity of extrapolation of results obtained using transgenic mice (the most studied model) to humans. As a result of interspecies differences in lipoprotein metabolism, it is especially remarkable that hamsters on high-cholesterol diets are among the small sized, non-primate models showing more similarities to human disease. Therefore, the research of risk factors related to diet-induced atherosclerosis could benefit from the study of this model.

In this context, driven by recent technological advances, such as mass spectrometry (MS) and bioinformatics, the ‘omics’-based approaches (such as genomics, transcriptomics, proteomics, and metabolomics) have allowed us to offer a global characterization, at a molecular level, of complex global biological systems and their changes in pathological processes. Complex networks comprising genomics, transcriptomics, and proteomics have previously been applied to study the functional basis of atherogenesis at the molecular level. These studies reveal the complexity of the networks underlying atherosclerosis, not only consisting of changes at a local level, but also involving integration of changes in the heart, vessels, and immune system, as well as other organs, such as the intestine, liver, adipose tissue, skeletal muscles, neuroendocrine regulatory tissues, kidneys, and pancreas.

Unlike genes and proteins, metabolites serve as direct signatures of biochemical activity and are therefore easier to correlate with phenotype. In this context, metabolomics and its derivative, lipidomics, have become a powerful approach that has been widely adopted for clinical diagnosis and opens a window to investigate how mechanistic biochemistry relates to cellular phenotype. Lipidomics, defined as the complete quantitative and molecular determination of lipid molecules isolated from biological samples, is a particular component of the metabolome. However, the different physicochemical properties of lipid species compared with water-soluble metabolites favour their separate analysis. Recent advances in MS and liquid chromatography (LC/MS) have strongly influenced the evolution of lipidomics. This advanced methodology makes it possible to identify and quantify hundreds of molecular species from the organism’s lipidome. The high number of molecules obtained in a metabolomic or lipidomic analysis requires specific statistical methodology. Multivariate statistics simplify the interpretation of the variation between samples that contain thousands of variables, reducing the variation to a two- or three-dimensional model.

Although two recent reports have applied metabolomics to the characterization of diet-induced atherosclerosis in hamsters, they did not include LC/MS, so the use of high-throughput chromatographic techniques, available by the use of ultra-high-pressure and sub-2 μm chromatographic columns, have not previously been applied. Furthermore, no direct comparison has been made between changes in circulating and tissue lipids. No direct study of lipidomic changes, with a focus on plasma and tissue changes, has previously been carried out in this particular model of diet-induced atherosclerosis. In humans, the use of tissue lipidomic analyses in atherosclerosis without chromatography has already revealed that in comparison between normal vessels and atherosclerosis-affected locations, the differential lipids comprise lysophospholipids and, mainly, cholesterol esters (CEs). Interestingly, CE species were highly enriched in plaque. These results indicate the importance of local accumulation of cholesterol species [and a decrease in the esterification by polyunsaturated fatty acids (PUFAs)] in atherosclerosis. Despite these previous data, inter-individual differences, as well as technical limitations, could be overcome by the application of these techniques. Furthermore, the elucidation of correlational analyses between the tissue and plasma lipidome, as well as the plasma metabolome, could lead to the establishment of novel pathogenic pathways that could provide a focus for further studies.

To shed light upon these questions, we analysed changes in the aortic and plasma lipidomes in a diet-induced model of early atherosclerosis. The results were compared with those of metabolomics in plasma, revealing the existence of several, previously unreported, changes in lipid and amino-acid metabolism, peroxisome proliferator-activated receptor γ (PPARγ) expression, and oxidative and endoplasmic reticulum (ER) stress. These also show a previously uncovered role for senescence-associated changes, such as β-galactosidase and the DNA damage response. Furthermore, to show the validity of this approach in the modelling of dietary influences in the development of atherosclerosis, we have measured the effect on the reported pathways of including a potential anti-atherogenic polyphenol extract in the diet.

2. Methods

2.1 Animals and diets

Atherogenesis was initiated in male Golden Syrian hamsters (Mesocricetus auratus; n = 15–16 per group) as previously described. Briefly, both control and atherogenic diets were maintained for 3 months. Animals were then fasted overnight, anaesthetized with 2.5% isoflurane in air, and killed by cardiac puncture in order to harvest the blood. The aorta was removed, and the aortic arch was separated from the descending aorta. In order to analyse dietary modulation of atheromatosis, an additional group ate the same diet as the atherogenic group, but supplemented with 0.2% grape seed extract (GSE; see Supplementary Data for further information). In a preliminary experiment to ascertain the bioavailability of GSE and its potential for changing the circulating metabolome and lipidome in a control situation, GSE extract was also used for 15 days in a similar group of hamsters that were not submitted to an atherogenic diet. All experimental procedures were approved by the Institutional Animal Care Committee of IRBLleida and conformed with Directive 2010/63/EU of the European Parliament.

2.2 Plasma lipid profile

The plasma lipid profile was evaluated in order to establish the effect of the high-fat diet. Plasma triglycerides (TGs), high-density lipoprotein (HDL), LDL, and total cholesterol (TC) content were evaluated by enzymatic colorimetric reactions using commercial kits (Spinreact, Girona, Spain; values in milligrams per decilitre: control group TGs = 67.36 ± 11.81, atheroma group TGs = 55.58 ± 4.85, control group HDL = 58.48 ± 5.18, atheroma group HDL = 71.67 ± 4.02, control group LDL = 60.64 ± 3.21, atheroma group LDL = 133.14 ± 7.82, control group TC = 182.27 ± 10.48, and atheroma group TC = 415.05 ± 18.85).

2.3 Lipidomic analysis

Lipids from descending aorta and plasma samples were extracted as previously described (see Supplementary Data for further information). Briefly, 7–12 mg of aortic tissue was homogenized with 50 μL of cold methanol. Then, 500 μL of chloroform and 187.5 μL of 0.7% of KCI were added. Proteins from plasma samples (25 μL) were precipitated with acetone. Then, 250 μL of methanol, 500 μL of chloroform, and
200 μL of 0.7% of KCl were added to the supernatants. For both aortic and plasma samples, the chloroform phase was separated, evaporated, and resuspended in chloroform:methanol (1:3, v/v). For both untargeted and targeted lipidomic analyses, lipid extracts were subjected to MS using an HPLC 1200 series coupled to an ESI-Q-TOF MS/MS 6520 (Agilent Technologies, Barcelona, Spain; see Supplementary Data for further information).

2.4 Metabolomic analysis
Plasma metabolites were extracted with methanol as previously described.\(^{19}\) Briefly, 30 μL of cold methanol was added to 10 μL of plasma to precipitate proteins. The supernatant was dried, resuspended in 50 μL of water, and applied to an HPLC 1200 series coupled to an ESI-Q-TOF MS/MS 6520 (Supplementary Data for further information).

2.5 Angiotensin-converting enzyme activity
Angiotensin-converting enzyme (ACE) activity in serum was determined using a fluorescence-based method previously described.\(^{20}\) Briefly, 25 μL of serum was mixed with 25 μL of 150 mM Tris-base buffer (pH 8.3) and 100 μL of ACE fluorescent substrate [0.45 mM of Abz-Gly-Phe(NO2)-Pro] in a 96-well microplate. After 10 min of incubation at 37°C, kinetic fluorescence (excitation/emission 355 nm/400 nm) readings were measured for 30 min (Supplementary Data for further information).

2.6 Immunohistochemistry of the aorta
Immunofluorescence was used to analyse the ER stress [measured using the reticulum chaperone protein disulfide isomerase (PDI)], as well as the presence of the scavenger receptor CD-36 in aortic tissue. Briefly, after permeabilization the slices were incubated at 4°C for 24 h either with rabbit anti-CD36 polyclonal antibody (ab78054; diluted 1:250; Abcam), or with mouse anti-PDI-ER Marker monoclonal antibody (ab2792; diluted 1:250; Abcam). The slices were incubated at room temperature for 1 h with the appropriate secondary antibodies. Mounted slices were examined under a Fluoview 500 Olympus confocal laser scanning microscope (Olympus, Hamburg, Germany; see Supplementary Data for further information).

2.7 Cell culture experiments
2.7.1 Transcriptional activity response of PPAR\(\gamma\) in human embryonic kidney (HEK293) cells
To assess gene expression, reporter gene methodology was employed. Briefly, HEK293 cells at a density of 10^5 cells per plate were transfected with 20 μg of PPRE X3-Tk-luc along with 10 μg of pHM829. Then, cells were exposed to plasma from the control, atherogenic, and GSE groups at a dose of 10% v/v with Advanced MEM and incubated during 5 h. The activation of PPAR\(\gamma\) was measured as the intensity of luminescence and normalized to the \(\beta\)-galactosidase activity (Supplementary Data for further information).

2.7.2 Induction of cell senescence in human mammary epithelial cells (HMECs)
Immunofluorescence was used to analyse the senescence-related protein γ-H2AX. Briefly, cells were incubated at 4°C with anti-γ-H2AX (phospho S139) antibody (ab2893; Abcam, Cambridge, UK) diluted 1:750 and detected with an appropriate secondary antibody. Cells were examined under a Fluoview 500 Olympus confocal laser scanning microscope (Supplementary Data for further information). The immunoreactive nuclei were counted using ImageJ software.\(^{21}\)

2.8 Statistical analysis
Non-targeted lipidomics and metabolomics analyses were carried out using MassHunter Mass Profiler Professional Software (Agilent Technologies, Barcelona, Spain), as detailed in the Supplementary material. Other statistical calculations were performed using SPSS software (SPSS Inc., Chicago, IL, USA). Normality of the distribution of variables was checked using the Kolmogorov–Smirnov test. Correlation between different parameters was evaluated using the Pearson correlation coefficient. Differences in the amounts of molecules between groups were analysed using Student’s unpaired t-test (when two groups were compared) or ANOVA with Bonferroni post hoc test (when more than two groups were compared). A level of \(P < 0.05\) was selected as the point of minimal statistical significance in every comparison. Individual data are presented in a database as Supplementary material.

3. Results
3.1 Intake of a high-fat diet produces a specific aortic lipidome signature
Given the importance of the aorta as a representative organ in early atherogenesis, we first analysed the effect of the high-fat diet on the whole aorta lipidome. We restricted those analyses to samples showing chemical evidence (increased cholesterol content) but lack of morphological evidence of atheroma formation, in order to restrict the study to early phases of atheroma formation. Using a non-targeted approach, we initially found 13 910 molecular features. After alignment and selection of those features that were present in at least 75% of the samples within the same group, we obtained 3986 molecular features. After a clustering analysis, the influence of diet was evident (Figure 1A and B). In order to explore the importance of the diet in determination of the lipidome, further multivariate statistics were used. As Figure 1C shows, both an unsupervised (PCA) and a supervised (PLS-DA) approach demonstrated that the clustering of the group was almost perfect (PLS-DA overall accuracy = 92%), suggesting that the diet was the main factor determining the aortic lipidome.

Using univariate statistics (Student’s unpaired t-test) we found 368 differential molecules (\(P < 0.05\), fold change \(> 2\)). Among these, we identified 18 (Table 1), based on retention time and exact mass. Specifically, the high-fat diet altered the levels of different lipid species in the aorta belonging to the categories of glycerophospholipids, sphingolipids, glycerolipids, free fatty acids (FFAs), and sterols.

3.2 Intake of a high-fat diet induces specific changes in plasma lipids, some of which are in common with the aorta
In order to obtain the inter-compartmental analyses, we analysed the plasma lipidome (19 998 molecular features; 2142 molecular features in at least 75% of the samples within the same group), demonstrating that the clustering effect of diet was lower than that present in the aorta (Figure 1D). Concerning differential lipid species between groups (150), we identified lipids belonging to the categories of glycerophospholipids, sphingolipids, glycerolipids, and FFAs (Table 2). Among these, ceramide (d18:1/24:1) and the PUFA docosahexaenoic acid (DHA) were increased in both atherogenic plasma and aorta. Furthermore, we found increased levels of plasma 1-palmitoyl-2-glutaryl-sn-glycero-3-phosphatidylcholine (PGPC), a phospholipid oxidation product, suggesting that there were higher levels of oxidative...
stress in the atherogenic group compared with the control group. Further search of other lipid oxidation markers in plasma and aorta revealed the presence of two oxysterols (the 7-keto-cholesterol and 5-cholesten-3β-ol-7-one) in plasma and two free fatty acid oxidation products [13,9-hydroxyoctadecadienoic acid (HODE) and 15-hydroxyeicosatetraenoic acid (HETE)], as well as the oxysterol cholesterol-5α,6α-epoxide in aorta, but no statistical significant differences were observed (data not shown).

### 3.3 High-fat diet induces changes in the plasma metabolome

In order to explore potential biomarkers further, we extended the analyses to the plasma metabolome. The Student’s unpaired t-test analyses revealed 480 differential molecules between the control and the atherogenic group. Among them, 57% were up-regulated and 43% down-regulated by the high-fat diet. After MS/MS analysis, we identified taurine (Figure 2A) and phenylalanine (Figure 2B), which were down-regulated with the high-fat diet. On the contrary, taurocholic acid was up-regulated with the high-fat diet, and is a potential biomarker of the process of atherogenesis (Figure 2C).

### 3.4 Intake of GSE is partly able to reverse the effect of a high-fat diet

In order to validate the chosen approach, we wanted to check the efficiency of a described cardioprotective vegetable extract, the GSE. We performed a preliminary analysis, in which the effect of the GSE on the plasma metabolome and lipidome was demonstrated in hamsters (Supplementary material online, Figure S1). Based on these results, GSE intake, even with a non-atherogenic diet, has an impact on the profile of the plasma metabolome and lipidome, demonstrating...
its potential bioaccessibility, bioavailability, and impact on host metabolism. Therefore, the same analyses were performed in a parallel group of hamsters that ate the atherogenic diet plus the GSE. The molecules for which GSE intake prevented the effect of the high-fat diet are shown in Table 3. Specifically, GSE was able to prevent the effect of high-fat diet on aortic concentrations of glycerophospholipids, glycerolipids, and sterol. Interestingly, the levels in the aorta of free cholesterol (FC), a known marker of early atherosclerosis, were the same in GSE group as in control animals, but lower than in the atherogenic group, suggesting a protective effect of this extract on atherogenesis. In plasma, GSE intake in animals on the high-fat diet led to control levels of lipid peroxidation, measured as PGPC. Moreover, the levels of one galabiosylceramide specie, as well as the levels of stearic and arachidonic acid, were decreased by GSE extract to control values. Finally, the potential plasma biomarker of atherogenesis, taurocholic acid, was also modulated by GSE intake.

### 3.5 Taurocholic acid as a plasma biomarker of atherogenesis

The next objective was to find potential plasma biomarkers that correlate with FC as well as FFA in the aorta as atherogenic markers. To this end, we performed a correlational analysis including cholesterol and oxidative stress biomarkers, as well as the metabolites identified, and several highly significant correlations were found (see Supplementary data for further information (Figure S2)).

Among these, the levels of taurocholic acid were positively correlated with the levels of FC in the aorta (Pearson correlation coefficient $r = 0.700$, $P < 0.004$), and taurocholic acid is therefore proposed as a potential biomarker of early atherogenesis (Figure 2D).

### 3.6 High-fat diet increases the activity of ACE and induces changes in PPARγ-related mechanisms

In order to characterize this approach further, we measured the plasma ACE activity and the ability of the plasma to modulate the transcription of PPARγ in HEK293 cells, because these are well-known regulatory mechanisms implicated in atherosclerosis. The ACE activity assay revealed that both atherogenic plasma and plasma from animals fed
with high-fat diet/GSE had an increased ACE activity compared with control plasma (Figure 3), suggesting higher risk of CVD.26

Previous studies have demonstrated27 an atheroprotective function of PPARγ, although its transcription is activated naturally by different FFAs (linolenic and arachidonic acid) and by lipid peroxidation products commonly found in plasma after a high-fat diet, among other factors.28,29 In the present work, we demonstrated an induction of PPARγ activation by plasma from early atherogenic animals (Figure 3A), indicating that some of the plasma components were inducing PPARγ-mediated gene expression. This effect was not observed using plasma from the GSE group, suggesting that the partial prevention of effects of the high-fat diet by this vegetable extract was crucial in the induction of PPARγ transcriptional activity. In order to clarify which plasma component could exert that effect, we incubated the cells with different concentrations of the potential biomarker, taurocholic acid (Figure 4A), and with the GSE (Figure 4B). Surprisingly, the analysis indicated an inhibitory effect of taurocholic acid at the highest concentration tested (5 mM) and an activating effect of GSE at the highest concentration tested (1 g/L). These data therefore suggest that the ex vivo effects of plasma in PPARγ activity could be a compensatory response by host-specific unknown factors in response to the direct effects of taurocholic acid and GSE in this system (respectively potentiating or decreasing PPARγ activity in vivo).

Furthermore, the induction of PPARγ was associated with higher levels of ER stress (as PDI immunofluorescence), as well as CD-36 expression in cells (Figure 3B), as previously described.30

3.7 High-fat diet induces changes in cell-senescence markers

Finally, because increased levels of glucosylceramides (a product of the well-known cell senescence-related enzyme, β-galactosidase (Figure S3)) were found in early induced atherogenic plasma samples, a DNA damage repair marker, the γ-H2AX, was studied. The analysis revealed that plasma from high-fat-diet-fed animals induced higher levels of γ-H2AX in endothelial cells in culture (Figure 3C). Most interestingly, the GSE diet was partly able to prevent this cell-senescence parameter. To shed light on the potential mechanism behind those changes, we studied the effects of both taurocholic acid and GSE on endothelial cell senescence. Specifically, these analyses demonstrated a pro-senescence effect of taurocholic acid at 1 μM (Figure 4C and Supplementary material online, Table 3 Atherogenesis-related molecules are reversed by intake of grape seed extract

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<tr>
<th>Location</th>
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<tr>
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<td>PC(O-19:1)</td>
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<td></td>
<td>Glycerolipids</td>
<td>TG(53:3)</td>
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<td></td>
<td>Sterols</td>
<td>5α-Tetrahydrocorticosterone</td>
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<td>3β-Cholane-3α,7α,12α-triol</td>
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<td>Free cholesterol</td>
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<td>Glycerophospholipids</td>
<td>PGPC</td>
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<td>Sphingolipids</td>
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<td>Taurocholic acid</td>
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Figure 2 Atherogenic diet induces lower levels of taurine (A) and phenylalanine (B) and higher levels of taurocholic acid (C) in plasma. (D) Taurocholic acid in plasma is correlated positively with aortic free cholesterol ($r^2 = 0.49, P < 0.003$). Curved lines represent 95% confidence intervals for values. $n_{\text{plasma}} = 13–16$, and $n_{\text{aorta}} = 6$. 

Table 3 Atherogenesis-related molecules are reversed by intake of grape seed extract
which was strengthened in the presence of a DNA alkylating agent, such as the methyl methanesulfonate (MMS; Figure 4D and Supplementary material online, Figure S5). These results strongly suggest that, besides being a good biomarker candidate, taurocholic acid could have a pathogenic role in early atherogenesis-associated endothelial cell senescence.

Reinforcing the complexity of the model, the presence of GSE did not affect cell senescence in vitro, assessed as the response to the induction of DNA double strand breaks (Figure 4E and Supplementary material online, Figure S6). Thus, the preventive effect of GSE probably involves indirect effects via the host metabolism, rather than a direct effect on endothelial cell senescence.

4. Discussion

The incidences of lifestyle-related cardiovascular and metabolic diseases continue to increase in Western populations. Atherosclerosis is principally associated with age and with a high-fat diet and other harmful habits that lead to hyperlipidaemia, hypertension, oxidative stress, and pro-inflammatory conditions, among other factors. Thus, in order to study the effect of a high-fat diet on the aorta and plasma lipidome we chose the hamster as the best small animal model. The hamster, contrary to wild-type mice and rats, is able to develop diet-induced atherogenesis. In a similar manner to humans, hamsters mainly transport cholesterol by (V)LDL (which contain only ApoB100), whereas in mice and rats the main part of the cholesterol is present in the HDL fraction. Furthermore, like humans, hamsters are the only small rodents that possess cholesteryl ester transferase activity, a crucial enzyme in lipoprotein metabolism. Therefore, the hamster was chosen as the diet-induced atherosclerosis model instead of one of the widely accepted transgenic mouse models, such as LDLR−/−, a model of familiar hypercholesterolaemia, or ApoE−/−, which mainly represents a familial disbeta-lipoproteinaemia. It should be remembered that diet is among the
most important modifiable factors involved in atherosclerosis. Consequently, experimental factors dealing with this involvement could shed light on the basic pathophysiological processes underlying atherogenesis. As a limit of the present work, we have not taken into account the potential importance of inflammatory factors and physical activity, or any other pathogenesis-modifying variables, which are not present in a diet-related pattern in this model.

Given that the aorta is one of the major targets of the experimental atherogenesis process, we started by studying the effects of the high-fat diet in aorta lipidome. We restricted the analyses to samples with an absence of morphologically detectable plaque, but with chemical evidence of it (increased cholesterol concentration). It is known that the accumulation of FC inside macrophages and endothelial cells is able to provoke structural changes in cell membranes, as well as cytotoxicity. In the particular case of macrophages, higher levels of FC are related to foam cell formation. Furthermore, an increased FC/cholesterol ester (CE) ratio has been described in human unstable atheromatous plaques. The importance of aortic FC as an atheroma initiation marker was reinforced by the study with the well-described anti-atherogenic GSE. The use of this extract prevented the accumulation of cholesterol in the aorta, validating the chosen approach.

The increased levels of DHA, a widely described cardioprotective PUFA, correlated positively with the levels of plasma TC and LDL-cholesterol, suggesting its presence as an evidence of a protective mechanism in the aorta induced by cholesterolaemia. Concerning glycerolipids, the analysis showed changes in both the quantity and the composition of TGs after the high-fat diet. Globally, these data are compatible with increased de novo synthesis in the atherogenic context.

It is well known that sphingolipids have been implicated in numerous intra- and extracellular signalling processes as both signalling molecules and secondary messengers. Their role in atherogenesis, as well as their presence in atheromatous plaque, is also well known. The presence of ceramide in atheromatous plaque is usually associated with the activity of the enzyme sphingomyelinase, especially when pro-atherogenic oxidized LDL are present. In the present study, we found increased the levels

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**Figure 4** The incubation of HEK293 cells with taurocholic acid at 5 μM (A) and grape seed extract at 1 g/L (B) induces lower and higher levels, respectively, of PPARγ transcription. (C) The incubation of taurocholic acid (TA) at 1 μM increases the DNA damage repair marker γ-H2AX in endothelial cells (HMECs) in culture. (D) This effect increases in the presence of a DNA-alkylating agent, such as methyl methanesulfonate (MMS). (E) On the contrary, the incubation with grape seed extract does not affect the senescence status in HMECs in presence of MMS. n = 4–6. Data shown are the means ± SEM of 4–6 different experiments. *P < 0.05 and ***P < 0.0001, statistically significant differences assessed using one-way ANOVA, with post hoc analyses.
of ceramide (d18:1/24:1), two precursors of ceramide synthesis (the 3-dehydrophosphogitine and the dihydrophosphogitine), and one derive [the (3-sulpho)Galβ-Cer(d18:1/20:0)], suggesting an induction of ceramide synthesis instead of sphingomyelinsa activity in the high-fat diet group.

In order to discover potential biomarkers for atheromatous plaque formation, we focused the study on the plasma lipidome and its comparison to changes in the aorta lipidome. Surprisingly, we discovered that a high-fat diet induced more changes in the aorta than in the plasma lipidome, suggesting that vessels exhibit a 'high-fat molecular memory'. Furthermore, we found only two lipid species that were significantly different in both plasma and aorta [the DHA and the ceramide (d18:1/24:1)], indicating the importance of studying tissue-based system biology in the pathogenesis of atherosclerosis. Interestingly, although the levels of total and LDL-cholesterol were higher in the atherogenic group than in the control group, we did not found a statistical difference in the levels of plasma, suggesting an increase of CE species. Intake of the high-fat diet induced higher levels of stearic, oleic, and arachidonic acids, and DHA, and decreased the levels of linoleic and myristic acid, indicating that a high-fat diet is able to modulate FFA metabolism in the plasma, as previously described. Furthermore, GSE intake reverted the levels of stearic acid, a saturated fatty acid associated with cardiovascular risk, as well as the pro-inflammatory and pro-athergenic arachidonic acid, reinforcing the cardiovascular protective effect of GSE intake.

Globally, both glycerophospholipids and sphingolipids were increased after high-fat diet intake. The increase in levels of phosphoethanolamines (PE) and phosphocholines (PC) could be explained by the higher levels of HDL lipoproteins. Furthermore, it has previously been described that patients with coronary artery disease have elevated levels of PE in the plasma, reinforcing the atherosclerosis model presented in this work.

It has previously been described that glycerophospholipids are basically transported in plasma via LDL particles in both healthy and hypercholesterolaemic patients. Additionally, higher levels of sphingomyelin were described in CVD patients. Therefore, the increased in ceramide and glyceroceramides could be explained by the increased levels of LDL-cholesterol, leading to higher risk of development of CVD. Interestingly, the levels of the PC oxidation product PGPC were increased in the atherogenic group with respect to the control group, suggesting a higher oxidative status and, therefore, a pro-atherogenic environment, which was reverted or prevented by GSE intake.

Interestingly, we found that three glucosylceramides were increased in atherogenic plasma samples, suggesting either higher β-galactosidase or lower glucosylceramidase activity. The increased levels of one ceramide, however, suggest a higher flux from lactosylceramide to glucosylceramide and, finally, to ceramide formation (see Supplementary material online, Figure S3). The fact that β-galactosidase activity is a well-known cell-senescence marker suggests an induction of cell senescence by the high-fat diet. This hypothesis was confirmed by cell culture experiments demonstrating that plasma from high-fat-fed animals induced higher levels of the DNA damage response marker, γ-H2AX.

The metabolomic analysis of plasma revealed lower levels of phenylalanine and taurine, reinforcing data showing that a high-fat diet is able to modulate amino acid metabolism. Additionally, the levels of taurocholic acid were increased in the atherogenic group and correlated strongly with FC in the aorta. Furthermore, the intake of anti-atherogenic diet (GSE) was also able to regulate the taurocholic acid levels, suggesting that taurocholic acid is a good candidate for a circulating biomarker of early atherosogenesis. Notably, there are other situations in which taurocholic acid in plasma is increased. While this manuscript was in revision, Devkota et al. showed that taurocholic acid was present in increased amounts in biliary secretions after a high-fat diet. Furthermore, it is known that blood concentrations of taurocholic acid increase in primary biliary atresia and during anti-retroviral drug treatment. Therefore, our data showing the presence of an increased level of taurocholic acid in experimental early atherogenesis, as well as the novel pathogenic pathways induced by this compound, may show promise as a new biomarker, but the above-mentioned limitations should be also taken into account.

Finally, and in order to characterize the model used further, we wanted to analyse the effect of plasma on two cardiovascular-related mechanisms, the renin–angiotensin system and the PPARγ pathway. The renin–angiotensin system is an ever-evolving endocrine system that contains considerable checks and balances on the production and catabolism of angiotensin peptides, most probably as a result of the manifold effects of angiotensin. Angiotensin II is a vasopressor hormone that is formed by the hydrolysis of angiotensin I by ACE. In addition to its hypertensive properties, angiotensin II is able to regulate cellular redox status (e.g. promoting LDL oxidation), dyslipidaemia, cell growth and proliferation, and production of chemokines and/or cytokines implicated in inflammation, all of which are important in the regulation of CVD. Thus, the increased levels of ACE activity in atherogenic and GSE plasma indicated that the high-fat diet could also promote the atherogenesis process, incrementing the levels of angiotensin II.

The PPARγ gene is found in vascular cells, and pharmacological agonists are considered to be potential anti-atherogenic drugs, because they can reduce the plasma TGs, increase the antioxidant enzymes, decrease the lipoxidative products, and finally reduce the atheromatous plaque in animal models. However, it has also been described that FFAs, such as linolenic and arachidonic acids, as well as oxidative stress products, such as prostaglandins, FFA derivatives (HODE and HETE species) and oxysterols (all of which are molecules commonly present in an atherogenic models), are natural ligands of PPARγ. The induction of PPARγ is directly related to monocyte maturation and, therefore, to CD36 expression in macrophages. In the present work, atherogenic plasma samples increased the expression of PPARγ and CD-36 expression, whereas dietary GSE reverted or prevented this effect, suggesting that linoleic and/or arachidonic acid, as well as PGPC or oxidative-related products, could induce this expression, in a close relationship with an ER stress marker, such as PDI. The induction of PPARγ by these products could be explained as compensative cell mechanisms in response to this diet and its harmful agents.

Collectively, these results reinforce the notion that system biology, when applied to vascular tissue and circulating samples, allows the proposal of a novel hypothesis for a given process. In this particular example, the process of early atherogenesis shows a great degree of molecular diversity, involving not only lipid metabolism, but also basic cell signalling and survival mechanisms.

Supplementary material

Supplementary material is available at Cardiovascular Research online.
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