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Metabolic Phenotypes of Carotid Atherosclerotic Plaques Relate to Stroke Risk: An Exploratory Study

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WHAT THIS PAPER ADDS

This study demonstrates that the metabolic signature of carotid plaque tissue from patients with cerebrovascular symptoms differs significantly from carotid plaque tissue derived from asymptomatic patients. This was achieved by a comprehensive metabolic profiling application using ultra performance liquid chromatography coupled to mass spectrometry. Enhanced downregulation of the β-oxidation pathway in symptomatic plaques is demonstrated for the first time. Metabolites associated with cell death were unaffected. The metabolic signatures identified show potential as differential diagnostic biomarkers for symptomatic plaques and may provide targets for pharmacotherapeutic intervention.

Objective: Stroke is a major cause of death and disability. That three-quarters of stroke patients will never have previously manifested cerebrovascular symptoms demonstrates the unmet clinical need for new biomarkers able to stratify patient risk and elucidation of the biological dysregulations. In this study, the utility of comprehensive metabolic phenotyping is assessed to provide candidate biomarkers that relate to stroke risk in stenosing carotid plaque tissue samples.

Method: Carotid plaque tissue samples were obtained from patients with cerebrovascular symptoms of carotid origin ($n = 5$), and from asymptomatic patients ($n = 5$). Two adjacent biological replicates were obtained from each tissue. Organic and aqueous metabolite extracts were obtained separately and analysed using two ultra performance liquid chromatography coupled to mass spectrometry metabolic profiling methods. Multivariate and univariate tools were used for statistical analysis.

Results: The two study groups demonstrated distinct plaque phenotypes using multivariate data analysis. Univariate statistics also revealed metabolites that differentiated the two groups with a strong statistical significance ($p = 10^{-4}$–10^{-5}). Specifically, metabolites related to the eicosanoid pathway (arachidonic acid and arachidonic acid precursors), and three acylcarnitine species (butyrylcarnitine, hexanoylcarnitine, and palmitoylcarnitine), intermediates of the β-oxidation, were detected in higher intensities in symptomatic patients. However, metabolites implicated in the process of cell death, a process known to be upregulated in the formation of the vulnerable plaque, were unaffected.

Conclusions: Discrimination between symptomatic and asymptomatic carotid plaque tissue is demonstrated for the first time using metabolic profiling technologies. Two biological pathways (eicosanoid and β-oxidation) were implicated in differentiating symptomatic from asymptomatic patients and will be further investigated. These results indicate that metabolic phenotyping should be further explored to investigate the chemistry of the unstable plaque, in the pursuit of candidate biomarkers for risk-stratification and targets for pharmacotherapeutic intervention.

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INTRODUCTION

According to the World Health Organization, stroke is a major cause of death and disability. Patients with cerebrovascular symptoms of carotid origin are at high risk of a subsequent imminent life-threatening stroke,[1] which declines with time after symptom onset. However, three-quarters of stroke patients will have been previously asymptomatic.[2] There is, therefore, an ongoing clinical need

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to identify biological markers that can stratify plaque rupture risk.[3] A recent metabolic profiling study in blood plasma demonstrated promising results for identifying patients with stroke recurrence.[4] A subsequent study using a lipidomic workflow to profile plaques reported successful discrimination between lipid signatures of the stable and unstable parts of the same plaque tissue, but not between plaque tissues obtained from symptomatic and asymptomatic patients.[5]

Metabolic phenotyping relies on the use of modern chemical analytical instrumentation to detect metabolic alterations in a biological system. To achieve a wide metabolome coverage, multiple methods or techniques are required.[6–8] Subsequent deconvolution and interpretation of the data is conducted through data-processing algorithms,[9] statistical analysis and modelling,[10,11] followed by molecular structure assignment and biological pathway mapping.[6,11,12]

Analysis of tissue samples can provide candidate biomarkers for *in vivo* imaging and guide further targeted biomarker discovery studies in matrices such as blood and urine. Most importantly — in contrast to blood plasma/serum samples which provide a more systemic view — tissue samples can provide clear, cell type-specific insight regarding biological mechanistic dysregulations.[11] However, the use of tissue for metabolic phenotyping can be challenging because of the additional steps required prior to analysis, such as tissue homogenisation[13] and metabolite extraction.[14] Methods with the ability to handle intact tissue function complementary to tissue extraction workflows,[15] and are preferred in translational clinical settings.[16]

It was hypothesised that stenosing carotid plaque tissue will exhibit a different metabolic signature according to patient symptomatic status. This is a pilot study employing comprehensive untargeted metabolic phenotyping methodologies to explore the ability to reveal metabolic signatures in stenosing carotid plaque tissue samples. Samples were obtained from patients who had recently (≤12 days) presented with a cerebrovascular event (high risk/symptomatic group), and from asymptomatic patients as a control group (low risk/asymptomatic group). Ultra performance liquid chromatography coupled to mass spectrometry (UPLC-MS) was the technique of choice used for the untargeted comprehensive metabolic profiling analysis.[6,17] Implicated mechanistic processes and candidate diagnostic signatures or metabolites, could function towards generating hypotheses and candidate biomarkers relating to plaque rupture and stroke risk.

METHODS

Patients

Atherosclerotic plaques were obtained from consenting patients and after research ethics committee approval (08/H0706/129), at the time of carotid endarterectomy surgery: five patients recently (≤12 days) symptomatic of cerebrovascular symptoms occurring in the territory of the ipsilateral carotid circulation, and five asymptomatic patients.

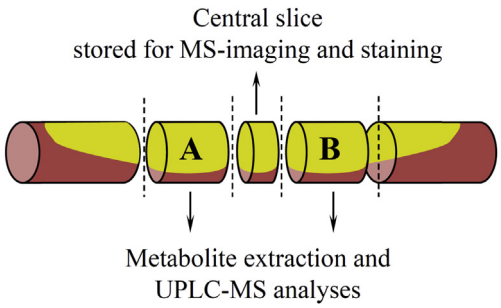

Figure 1. A schematic demonstrating carotid plaque tissue sectioning and allocation. Segments A and B underwent separate metabolite extraction and analysis. The central segment was stored for future analyses using MS-imaging and staining. The yellow colour represents the stenosing plaque. UPLC = ultra performance liquid chromatography; MS = mass spectrometry.

Patients were considered asymptomatic if they did not have any focal neurological symptoms pertaining to the anterior circulation of the cerebral hemisphere ipsilateral to the index carotid stenosis within the 6 months prior to carotid endarterectomy. The patients with asymptomatic carotid stenosis in this study had never experienced focal neurological symptoms at any time point prior to their carotid endarterectomy. There was no post-operative mortality among the patients enrolled. One symptomatic patient developed a post-operative haematoma which required operative evacuation on the first post-operative day. Patients' demographics can be found in Supplementary Material Table S1.

Sample preparation

Three transverse segments of stenosing carotid plaque tissue were obtained from each sample. The central slice was stored for imaging and staining purposes. The two slices flanking the central slice were placed into separate bead beating tubes, for tissue lysis and metabolite extraction. Two consecutive extractions were performed: for polar compounds (aqueous extracts), and lipophilic compounds (organic extracts).[6] A detailed description of sample preparation is presented in the Supplementary Methods. A schematic illustration of the tissue sampling strategy is given in Fig. 1.

UPLC-MS analyses data processing and statistics

An untargeted lipidomics reversed phase (RP)-UPLC-MS analysis was applied to the organic extracts.[6] An untargeted polar metabolic phenotyping method was employed for analysing the aqueous extracts using hydrophilic interaction liquid chromatography (HILIC)-UPLC-MS.[6] These two UPLC-MS methods combined can cover analytes in a range of physicochemical properties, maximising metabolome coverage.[6] Samples were analysed in both positive and negative electrospray ionisation (ESI) modes. The two polarity modes generate complementary information resulting from preferential ionisation of metabolites (diminished ionisation can reduce sensitivity) according to their