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Simultaneous detection of five one-carbon metabolites in plasma using stable isotope dilution liquid chromatography tandem mass spectrometry

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ABSTRACT

Disturbance in one-carbon (1-C) cycle occurs due to nutritional deficiencies (vitamin B12/folate) or specific genetic polymorphisms. This leads to altered levels of key 1-C metabolites such as SAM (*s*-adenosyl methionine), SAH (*s*-adenosyl homocysteine), methionine, homocysteine and MMA (methyl malonic acid). These 1-C metabolites are determinants of cellular methylation potential and epigenetic modifications of DNA which impairs metabolic pathways in several pathological diseases and developmental programming. Though methods were able to measure these analytes only independently, none of the methods detect simultaneously. Therefore we developed a method to measure these five 1-C metabolites in a single run using liquid chromatography tandem mass spectrometry (LC–MS/MS). We used stable isotopes dilution LC–MS/MS to measure the 1-C metabolites in human plasma. Blood samples were collected from pregnant women ($n = 30$) at early gestation in the ongoing, multicentre, prospective PRiDE study. Linearity exhibited across the calibration range for all the analytes with the limit of detection (LOD) of 1.005 nmol/l for SAM, 0.081 nmol/l for SAH, 0.002 μmol/l for methionine, 0.046 μmol/l for homocysteine and 3.920 nmol/l for MMA. The average recovery for SAM was 108%, SAH—110%, methionine—97%, homocysteine—91% and MMA—102%. The inter-assay CV for SAM was 7.3, SAH—5.6%, methionine—3.5%, homocysteine—7.0% and MMA—4.0%. The intra-assay CV for SAM was 8.7%, SAH—4.7%, methionine—5.4%, homocysteine—8.1% and MMA—6.1%. Pregnant women at early gestation with low B12 levels had significantly higher homocysteine, MMA, lower levels of methionine, SAM and SAM:SAH ratio and higher triglycerides. We developed a simple and rapid method to simultaneously quantify 1-C metabolites such as SAM, SAH, methionine, homocysteine and MMA in plasma by stable isotope dilution LC–MS/MS which would be useful to elucidate the epigenetic mechanisms related in the gene–nutrient interactions.

1. Introduction

One-carbon (1-C) metabolism comprises a series of biochemical reactions in which methyl groups are generated or utilized in the presence of multiple enzymes, vitamins and cofactors. Perturbations in the 1-C metabolism either due to deficiencies in the 1-C substrates/co-factors or polymorphic variances in genes encoding 1-C enzymes leads to increased or decreased methyl groups resulting in altered methylation of DNA [1]. Vitamin B12 is a key component of the 1-C cycle and data from both human and animal studies support that maternal and fetal vitamin B12 availability has important influences on health outcomes, including fetal growth [2], fertility [3,4], long-term cardio-metabolic disease risk [5,6], neurodevelopment [7] and altered risk of cancer [8]. Vitamin B12, an essential cofactor for the enzyme methionine synthase transfers the methyl group from 5MTHF (methyltetrahydrofolate) to homocysteine to form methionine. Methionine, the direct precursor of *S*-adenosyl methionine (SAM) is metabolized into *S*-adenosyl homocysteine (SAH) by generating a methyl group, which is the universal methyl donor required for methylation of DNA, protein

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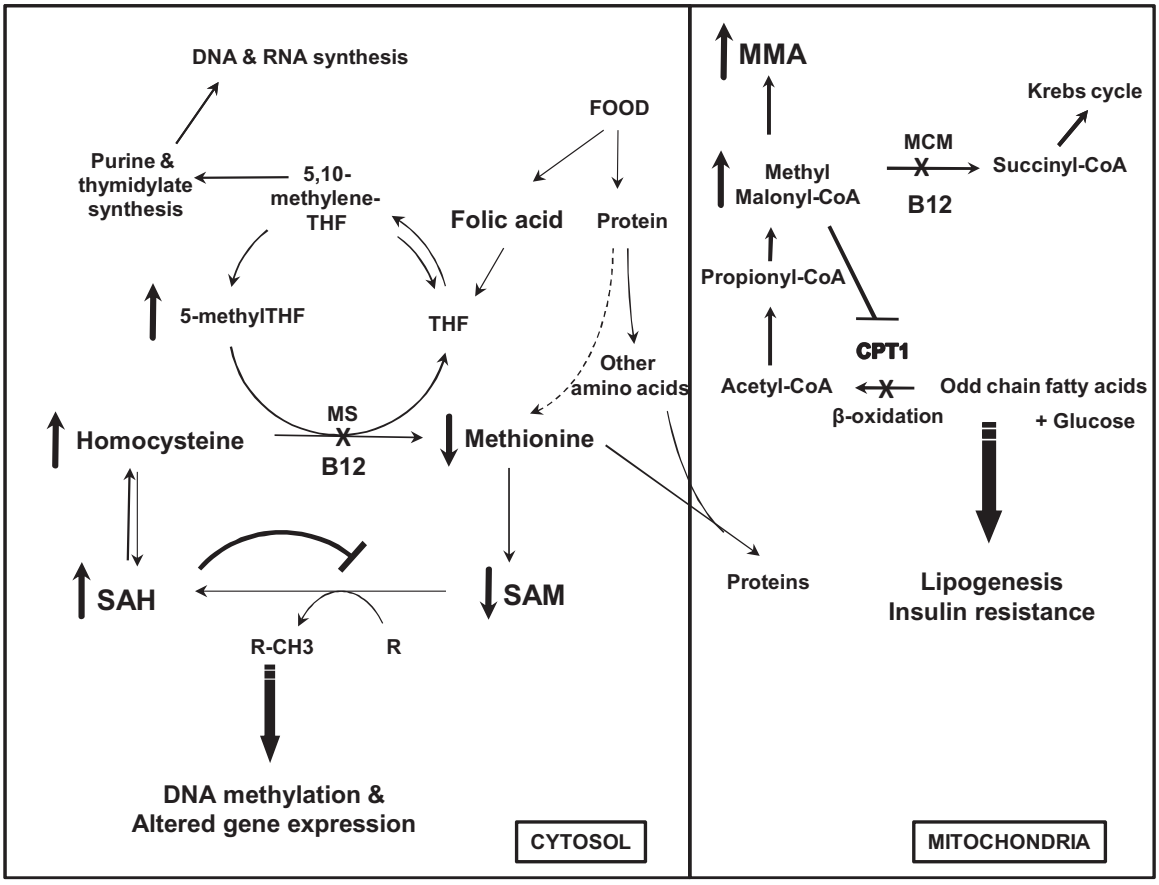

Fig 1. Overview of the one-carbon metabolism pathway and its implication in DNA methylation. Micronutrients (vitamin B12, folate) act as cofactors in the enzymatic reactions in cycle. Abbreviations: SAH—*s*-adenosyl homocysteine; SAM—*s*-adenosyl methionine; THF—tetra-hydrofolate. 5-MTHF—5-methyltetra hydrofolate; MS—methionine synthase; MCM—Methyl malonyl co-A mutase; CPT1—Carnitine palmitoyl transferase 1.

and lipids. SAH is further hydrolysed to homocysteine to complete the cycle. The generated methyl groups from this cycle is required for the DNA methylation, a major epigenetic process implicated in the regulation of gene expression levels [9,10]. Hence, when vitamin B12 is deficient, folate becomes trapped as 5-MTHF, regeneration of methionine and SAM is inhibited and the concentrations of homocysteine and it metabolites (SAH) are increased (Fig. 1). A reduced SAM:SAH ratio (called the methylation index) is indicative of global DNA hypomethylation.

Vitamin B12, beside its role in the 1-C cycle, also acts as a cofactor for the mitochondrial enzyme methylmalonyl co-A mutase, which catalyzes methylmalonyl Co-A (MM-CoA) to succinyl Co-A. Low vitamin B12 causes higher MM-CoA levels, which in turn inhibits carnitine palmitoyl transferase-1 (CPT-1), the rate-limiting enzyme for fatty acid β-oxidation, thereby increasing lipogenesis [5,11]. Thus vitamin B12 deficiency influences both folate-dependant methylation reactions and mitochondrial lipid metabolic pathways.

Therefore it is evident that vitamin B12 deficiency leads to disruption of the 1-C cycle and alters the levels of 1-C metabolites such as methionine, homocysteine, SAM, SAH and MMA. Animal studies deprived of periconceptual methyl groups showed biochemical deficiency of B12, folate, methionine, increased homocysteine and reduced SAM:SAH ratio [12]. A study in fetal human livers showed that maternal vitamin B12 together with smoking associated with altered levels of enzyme transcripts and cofactors involved in 1-C metabolism and altered methylation of IGF2 gene [13]. Recently we demonstrated that adipocytes cultured in low B12 condition had higher homocysteine, SAH and reduced SAM, SAM:SAH ratio and associated hypomethylation of cholesterol transcription factor (SREBF1 &LDLR) [14]. In addition, our clinical study displayed that maternal B12 deficiency associated with higher cord blood lipid profile [15]. These studies thus provide evidence that in addition to micronutrient (vitamin B12/folate) deficiencies, 1-C metabolites are determinants in programming the cardio-metabolic phenotype in offspring. Therefore along with the routine clinical measurements of vitamin B12 and folate, simultaneous determination of 1-C metabolites is an important tool not only to identify the subclinical deficiency of vitamin B12 (homocysteine and MMA) but also to evaluate the cellular methylation index (methionine, SAM, SAH) which regulates the epigenetic process and complex transcriptional hierarchies that define the functional phenotypes.

Though various analytical methods using HPLC and LC–MS/MS [16–20] have been developed to measure 1-C cycle metabolites, only two or three analytes of the cycle are detected, but no methods are available to simultaneously investigate all the five 1-C metabolites such as methionine, SAM, SAH, homocysteine and MMA. Moreover, independently analysing these analytes are expensive, sensitive to degradation, involves an additional derivatization step for sample preparation [16,20] and the physiological levels of SAM and SAH are quiet low [16]. The average reference ranges for these 1-C metabolites were SAM—71–168 nmol/l, SAH—8–26 nmol/l, methionine—6–53 μmol/l, homocysteine—5.4–13.9 μmol/l and MMA—73–271 nmol/l [21,22]. Hence a method with simultaneous measurement would be much more reliable and cheaper in a clinical setting. Therefore, we developed a simple, rapid and easy purification method to measure these analytes in a single run