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The Application of a Chemical Determination of N-Homocysteinylation Levels in Developing Mouse Embryos: Implication for Folate Responsive Birth Defects

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Abstract

Elevated homocysteine levels have long been associated with various disease states, including cardiovascular disease and birth defects, including neural tube defects (NTDs). One hypothesis regarding the strong correlation between these various disorders and high levels of homocysteine is that a reactive form of this small molecule can attach to mammalian proteins in a phenomenon known as homocysteinylation. These posttranslational modifications may become antigenic, or may even directly disrupt certain protein function. It remains to be determined whether dietary influences that can cause globally increased levels of circulating homocysteine confer negative effects maternally, or may otherwise negatively and materially impact the metabolic balance in developing embryos. Herein we present the application of a chemical method of determination of N-homocysteinylation to a set of neural tube closure stage mouse embryos and their mothers. We explore the uses of this newly-described technique to investigate levels of maternal and embryonic N-homocysteinylation using dietary manipulations of onecarbon metabolism with two known folate responsive neural tube defect mouse models. The data presented reveals that although diet appeared to have significant effects on the maternal metabolic status, those effects did not directly correlate to the embryonic folate or N-homocysteinylation status. Our studies indicate that maternal diet and embryonic genotype most significantly affected the embryonic developmental outcome.

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1. Introduction

High levels of plasma homocysteine have historically been associated with cardiovascular disease, stroke, elevated risks of bone fractures, birth defects, and neurodegenerative disorders[1] [2], although any causality due to this correlation continues to be hotly debated in the literature [3]. Due to the large proportion of the human population affected by any number of these pathologies, there is a need to better understand the potential link between homocysteine levels and the numerous disease states with which aberrant homocysteine levels has been associated. A promising hypothesis regarding the deleterious effects observed when individuals exhibit high homocysteine levels is the natural reactivity of homocysteine's metabolites. Homocysteine is a non-protein incorporating amino acid[4] and a cyclic by-product of DNA, protein, or lipid methylation by S-adenosyl methionine. With a single carbon unit donation by 5-methyltetrahydrofolate, homocysteine is converted into its precursor, methionine, which can be used in protein synthesis or again for any number of methylation reactions. 5-methyltetrahydrofolate is a bioactive form of vitamin B9, a water-soluble class of vitamins known as the folates[5]. The folates are responsible for mediating any single carbon donation including those involved in DNA base synthesis, the conversion of homocysteine to methionine, and the methylation of proteins, lipids, and DNA. Because of the place of homocysteine in one carbon metabolism, diets high in methionine and low in folate may well contribute to an increase in corporal levels of homocysteine. This direct relationship between homocysteine and folate emphasizes the potential roles that homocysteine may have in folate responsive conditions such as susceptibility to the induction of neural tube defects (NTDs). There is more than one metabolic fate of homocysteine, other than being returned to methionine with a methyl donation from 5-methyltetrahydrofolate. Homocysteine can be converted to cysteine via a trans-sulfuration reaction, or to homocysteine thiolactone using methionyl t-RNA synthase, thus preventing or greatly ameliorating protein misincorporation[6],[7].

In many disease models, high homocysteine levels have been related to increased immune responsiveness [8] [9]. One hypothesis relating high homocysteine levels to an increased immune response and inflammation is based on the chemical reactivity of homocysteine thiolactone. This natural metabolite that remains in balance with homocysteine in the mammalian system can covalently bond either exposed cysteines or lysines of proteins. It has been observed that approximately 80% of all homocysteine in the human system is protein bound [10]. There is no known mechanism in place for removing a so-called homocysteinylation event from a protein [11]. It is theorized that when these homocysteinylation events occur, plaques form or proteins become autoantigenic [12]. Since the system can never rid itself of one or more homocysteinylation events on proteins, high levels of homocysteine will, over time, increase the total amount of homocysteinylation of bodily proteins, especially those that do not have a high turnover rate [12],[13].

A major issue that has compromised this field of research is the difficulty of the empirical determination of the respective amounts of homocysteine, and simultaneously distinguishing between free homocysteine and S-homocysteinylation and N-homocysteinylation events. Mass spectrometry methods allow for the determination of many different post-translational events[14]. Mass spectrometry, however, is a technically difficult, intensive, and expensive

method to determine the modifications of only one protein at any given time. Although such an approach is highly sensitive, the methodology is challenging to utilize in determining the entire sum of homocysteinylated proteins in any given sample. There have been some reports of an antibody that binds only homocysteinylated lysines[15], but the dissemination of these studies has been scarce[15]. Also[16], as with many polyclonal antibodies, there is generally exhibited a high rate of non-specific binding. Recently, the literature has reported colorimetric methods, but those methods apparently produce whole determinations of free homocysteine[17], and this method cannot be adapted to distinguish protein-bound homocysteine. There is a critical need for an accurate, high-throughput method to determine N-homocysteinylated proteins. The idea that high levels of N-homocysteinylated proteins are directly linked to a multitude of common disease states needs to be explored. Lacking an appropriate assay with which to probe this question has greatly limited a field of research that could be applicable to many of the world's most common disorders.

Elevated homocysteine levels have also been shown to have teratogenic effects during pregnancy. [18] [19] There appears to be a relationship between higher plasma homocysteine levels in women who have pregnancies affected with neural tube defects (NTDs) than women with unaffected pregnancies[20],[21],[22]. It has also been noted that there is a strong linkage between an increase in maternal inflammation and the risk for NTDs[23]. Disease states such as diabetes and obesity are well-established mediators of increased inflammatory states[24],[25]. Mothers who have diabetes during pregnancy are seen to have a 2- to 10-fold increase in the rates of NTDs among their offspring[26]. Even mothers who do not have diabetes yet but are clinically obese (BMI over 29) are nearly twice as likely to have pregnancies affected with NTDs [27]. In general, NTDs affect between 1 and 10 in every 1000 pregnancies, depending on regional and ethnic groups being examined. Although it is commonly accepted that folic acid (Vitamin B9) can decrease the risk of an NTD-affected pregnancy, the presence of high levels of folic acid does not necessarily protect all pregnancies from adverse outcomes. Nor is it currently known why folic acid supplementation has been relatively successful in preventing so many NTDs. What has been clearly established is that NTDs are multifactorial in origin; there are genetic, dietary, and environmental factors at play in their etiology. In fact, some single mutations in key genes do not manifest with NTDs in mice, but when two mutations are introduced in two different genes, NTDs are observed [28]. In addition, it has been reported that some genetic mutations do not manifest with NTDs unless there is an additional stress imposed, such as exposure to certain environmental factors[29] [30].

One potential mechanism explaining the efficacy of folic acid supplementation in the prevention of NTDs deals with the reduction in homocysteine levels observed when individuals exhibit higher folate levels[31],[32]. It is possible that supplementation with folate during pregnancy may reduce the maternal homocysteine levels enough to promote more favorable pregnancy outcomes. Although mechanistically there is not one single answer as to why homocysteine is such an important indicator of pregnancy outcome, it is thought that improper regulation of the methionine cycle in the presence of low folate levels may be a key factor[33]. The methionine cycle produces S-Adenosyl Methionine (SAM), the major methyl donor for proteins, lipids, and DNA. Misregulation of this metabolite may drastically change the proteome through posttranslational modifications and epigenetic

regulation. These changes could act concurrently with the negative effects initiated by homocysteine thiolactone and N-homocysteinylation, creating conditions that compromise proper embryonic development. However, it is not currently known whether maternal folate and homocysteine status directly affect the folate and homocysteine status of the developing embryo, or whether an embryonic balance between folate and homocysteine is established by the time of neural tube closure. To date, there have been limited published data and mostly pure speculation on the correlation between maternal homocysteinylation levels and fetal homocysteinylation levels, as no published studies have directly compared maternal homocysteinylation levels to embryonic homocysteinylation levels during development, specifically, during the critical period of neural tube closure.

There are multitudes of methods with which elevated homocysteine levels can be achieved in mouse models. The easiest ways of inducing hyperhomocysteinemia are by spiking the administered diet with methionine, providing drinking water with homocysteine itself, or having a diet deficient in folate and vitamin B12 [34]. In addition, there are some genetic mutations that can cause a state of hyperhomocysteinemia. The genes manipulated to create high homocysteine levels have commonly been associated with one-carbon metabolism due to the central location that homocysteine holds in these metabolic cycles. The proton coupled folate transporter (PCFT) is one of the proteins responsible for transport of folates into cells at a low pH. PCFT is thought to be one of the major transporters of folates absorbed from the digestion of food in the intestine[35]. Elimination of Pcf^t in mice results in folate deficient conditions and high levels of total homocysteine and N-homocysteinylation [35] [36]. Methylene tetrahydrofolate reductase (MTHFR) is the enzyme responsible for the conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate. The product of this conversion, 5-methyltetrahydrofolate, is responsible for the one carbon unit donated to homocysteine so that it can be recycled into methionine. Mutations in this enzyme are associated with human cases of hyperhomocysteinemia[37] and some mutations in this gene have been associated with increased risk for NTDs in human epidemiological studies[38][39]. Mice that lack either one or both copies of MTHFR have up to a 10-fold increase in total homocysteine levels and are seen to have significantly higher levels of total N-homocysteinylation [36, 40]. The use of two known mouse models that have both elevated total homocysteine and elevated N-homocysteinylation levels allowed for the validation of the method of N-homocysteinylation presented here.

Herein we present the application of a novel technique of the chemical determination of N-homocysteinylation[41] that has been utilized to address the hypothesis that N-homocysteinylation can contribute to NTD susceptibility. The assay has been performed in murine embryos collected directly following the period of neural tube closure. The use of multiple mutant mouse strains exposed to differing one carbon defined test diets enabled us to further evaluate how both diet and genotype can influence pregnancy outcomes with respect to N-homocysteinylation, and how this post-translational modification fits into the complexities of neural tube development. Manipulation of the maternal diet included a high folate diet (10 ppm FA), a normal control diet (2 ppm FA), and a high methionine diet (1% added L-Methionine) intended to drive the formation of homocysteine. Two different genetic factors are investigated here: the folate responsive NTD mouse models of Lrp6 Cd

and Folt1. The Folt1 mouse model[42] is a well-characterized mutant where the nullizygous embryos present with NTDs, whereas the heterozygous mice are indistinguishable from wildtype. This mouse model knocks out one of the high affinity folate receptors that is heavily expressed in the placenta[43]. The mice completely lacking Folt1 can be rescued with maternal folate supplementation and appear to be completely normal in adulthood. A second folate responsive NTD mouse model is the Lrp6 Cd point mutation mouse. This NTD model was originally referred to as the “crooked tail mouse”, based on its phenotype. Molecular characterization revealed this to be the result of a single substitution in the Lrp6 gene[44]. When both copies of the Lrp6 gene have the Cd point mutation, mice exhibit a high penetrance of exencephaly, profound runting, and skeletal defects [45]. The exencephaly response frequency is decreased by nearly 55% when the Lrp6 Cd dams are supplemented with folic acid[45]. Previous studies have shown a digenic interaction when Folt1 and Lrp6 Cd heterozygous mice are crossed, resulting in double heterozygous animals having a 44% penetrance of NTDs, whereas either single heterozygous mutant shows no NTDs (Finnell and Ross, unpublished data). Using Folt1 and Lrp6 single heterozygous mating pairs of mice on different diets enables us to further explore the interaction between these two very different NTD candidate genes and maternal metabolic environments with respect to the etiology of NTDs.

The chemical determination for N-homocysteinylation described by Zang, et al[41], was modified here to interrogate a large set of biological samples. This new approach represents an easily-accessible and highly specific assay for a stoichiometric quantification of N-homocysteinylation events. The assay relies on the specific chemical reaction between either beta or gamma aminothiols and aldehydes under reducing conditions[46],[41]. The flexibility of this assay allows for either a solid-state aldehyde to identify N-homocysteinylation events, the creation of a gamma aminothiol, or a tagged aldehyde, such as the biotinylated aldehyde used here. To our knowledge, this new and highly specific technique has not yet been applied to any set of biological samples or to address a compelling medical issue such as the role of maternal inflammatory responses on the susceptibility to the induction of NTDs. As such, we chose to build upon earlier research using known mouse models to expand the uses of this assay to whole tissue samples, then adapting the chemistry to a dot blot assay for high throughput analysis of a set of mouse embryos. This approach enables a novel exploration of the multifactorial nature of NTDs. In this study we present metabolic data from neural tube closure stage embryos that are the product of different dietary and genetic influences.

2. Materials and Methods

2.1 Animals and Experimental Diets

Female Folt1 mice on an LM/Bc background, and Lrp6 Cd mice on a AJ background were maintained on a 12-hour light/dark cycle in the Dell Pediatric Research Institute Vivarium. At 6 weeks of age, female Folt1 (+/-) mice were placed into one of three experimental treatment groups consisting of a specific amino acid defined diet for a 6-week washout period. After diet washout, 4 dams were caged with one male and vaginal plugs were checked daily. Lrp6 Cd (Cd/+) males of comparable age were provided milled chow

(PicoLab Diet 20 no. 5053, Lab Diet) until being exposed to the females. The three amino acid defined experimental diets (Research Diets) used included: a control diet (2 ppm FA), a high folate diet (10 ppm FA), and a high methionine diet (1% L-Methionine). PCFT and MTHFR animals were housed in the same facility, maintained as breeding pairs and fed milled chow *ad libidum*. All animals were maintained according to institutional rules (IACUC protocols AUP 2013-0051 and AUP 2014-00276).

2.2 Animal Procedures

At day 9.5 post conception, blood was collected from the dams by a submandibular puncture in K₂EDTA coated collection tubes (BD Biosciences) and spun for 10 minutes at 3500XG for plasma separation. Dams were sacrificed with CO₂ asphyxiation followed by cervical dislocation and embryos were harvested in PBS and preserved on ice. Yolk sacs were collected from the embryos for genotyping and embryos were frozen at -80°C until further assays were performed.

2.3 Genotyping

Dams, sires, and embryos were genotyped for the Fcfr1 and the Lrp6 Cd point mutation. The Fcfr1 mutation was detected using wildtype primers 5'-AAG TGC AAG GCT GCA TGT GG-3' and 5'-CAT TCC GAT GTC ATA GTT CCG C-3', where the Fcfr1 null mutation was detected using the mutant primer set 5'-ATC GCC TTC TAT CGC CTT CTT GA C-3' and 5'-TGC ATT CCG ATG TCA TAG TTC CG-3'. The Lrp6 Cd mutation was detected using a PCR amplification of the Lrp6 gene followed by detection of the point mutation with a restriction digest. The Lrp6 gene was amplified with the primer set 5'-TGA CAA GCC ATC AAG CAG AG-3' and 5'-GCT CAG AGG CTA TGT GAA CCA-3'. The point mutation was detected with a restriction digest using Bsp1 (New England Biolabs). The MTHFR mutant was detected with 5'-GAC TAC CTG GCT ATC CTC TCA TCC-3' and 5'-AGC CTG AAG AAC GAG ATC AGC AGC-3', whereas the wildtype allele was detected with 5'-GAC TAC CTG GCT ATC CTC TCA TCC-3' and 5'-GAA GCA GAG GGA AGG AGG CTT CAG-3'. The PCFT mutant gene was detected using 5'-GCA GCG CAT CGC CTT CTA TC-3' and 5'-CTT GAC CAC AAC TGT CCA TGT GC-3'. The wildtype allele was detected using 5'-CCC AAC TCC AAA FCF CAG GTT CAT-3' and 5'-TCC AGA TGG GAA AGA AGA GGT-3'.

2.4 Folic Acid Measurements

A competitive ELISA assay was used to determine the concentration of total folates present in plasma and whole embryo tissue, as previously described (Cabrera). In short, tissue or plasma was boiled in Tris Buffered Saline with 0.1% Tween-20 and 1% w/v ascorbate buffer to release the folates into the buffer. The samples were subsequently spun down, the supernatant was collected, and then brought to a neutral pH with NaOH. The folate in these samples was then detected with a competitive binding method previously described [47]. In short, 96-well high binding plates (Immulon, Thermo Fisher Scientific Inc.) were printed with 25ng of bovine folate binding protein (Sigma Aldrich). The protein was allowed to adhere to the plate overnight at 4°C. Plates were then washed three times with Tris Buffered Saline (TBS) and 0.05% Tween 20 pH 8, to remove protein not bound to the plate.

Supernatant containing the released folates and a standard amount of an HRP tagged folate (Vitros Immunodiagnostics, Ortho-Clinical Diagnostics Inc) were incubated with the protein spot for one hour in the dark. Plates were then washed three times with TBS and 0.05% Tween 20 followed by two more washes with TBS. The amount of HRP-Folate binding was determined using ELISA Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific Inc). The plates were imaged using the Quansys Biosciences Q-View Imager and Software. Pixel intensities represent an inverse relationship with the amount of folate present in the sample. Qualitative data was then used to compare the amounts of folate between samples.

2.5 N-Homocysteinylation assay

Embryos were lysed in RIPA buffer with protease inhibitor cocktail tablets rocking at 4°C for 4 hours. Protein was then frozen and stored at -80°C to complete lysis. Bradford assays were performed to quantify total protein amounts. Lysates were incubated with 200 µM biotin aldehyde (Peptides International) in 50 mM citric acid (Sigma Aldrich), and 500 µM TCEP adjusted to pH 3 in the dark, at 25°C, for 14–16 hours. Samples were segregated by a 10 kDa molecular weight cut-off membrane (Pall), to exclude nonreacted substrate from the analysis.

2.6 Dot blot methods

1µL of sample was spotted onto a nitrocellulose membrane and allowed to dry. Membranes were blocked in 5% powdered milk solution for 1 hour at room temperature. Membranes were probed with an anti-streptavidin probe (1:10000, LiCor). Detection of spot intensity was performed using the LiCor Odyssey and associated software. 96 well data analysis spot function was used to determine the average integrated pixel intensity for each sample.

2.7 Mass Spectrometry

Samples were prepared using affinity chromatography as previously described[41]. In short, bovine serum albumin (Sigma Aldrich) was reacted with homocysteine thiolactone in 0.1M sodium phosphate and 20mM EDTA. The reacted protein was separated from the reactants using a Centricon membrane (Millipore). The protein was enzymatically digested with trypsin (Promega) overnight at 37°C in 95mM ammonium bicarbonate, 1mM TCEP, and 10% acetonitrile. The trypsin was inactivated with formic acid and excess solvent was evaporated. The trypsinized product was then allowed to bind to POROS 20 AL (Applied Biosciences) beads with aldehyde substituents. Beads were washed with increasing concentrations of NaCl and acetonitrile.

Homocysteinylation of lysines were eluted with O-methylhydroxylamine [41]—The trypsin digested BSA was analyzed by LC-MS/MS using the Proxeon Easy-nLC II coupled to the Thermo Velos Pro as previously described [48]. Prior to HPLC separation, the peptides were desalted using Millipore U-C18 ZipTip Pipette Tips following the manufacturer's protocol. A 2 cm long × 100 µm I.D. C18 5 µm trap column (Proxeon EASY Column) was followed by a 75 µm I.D. × 15 cm long analytical column packed with C18 3 µm material (Dionex Acclaim PepMap 100). Buffer A was composed of 0.1% formic acid in water and Buffer B 0.1% formic acid in acetonitrile. Data was acquired for 38 min using an HPLC gradient of 5% B to 45% B over 30 min with a flow rate of 300 nl/min. The data

dependent method consisted of an MS scan followed by CID MS/MS of the top 10 precursor ions. The mass range was m/z 300–1700, with 2.0 amu isolation width for MS/MS, collision energy 35, and dynamic exclusion after 2 counts for 60 sec. Raw data was processed using SEQUEST embedded in Proteome Discoverer v1.4.0.288 using the following parameters: full trypsin digest with maximum 2 missed cleavages and variable modifications oxidation of methionine and lysine homocysteinylation (mass addition C_4H_7NOS , monoisotopic mass 117.024835, average mass 117.1695), searching the SwissProt all species database dated July 22, 2013 (455,256 entries). The mass accuracy was set to 1.2 Da average mass for precursor ions and 0.8 Da monoisotopic for fragment ions. A decoy database was generated from the SwissProt database and used for calculating peptide probabilities. X!Tandem (GPM, version CYCLONE (2010.12.01.1) database searches were performed embedded in Scaffold 4 Q+ v.4.3.0 (Proteome Software) on a subset database using the same search parameters as SEQUEST with additional variable modifications of pyroglutamic acid modification of glutamine on peptide N-terminus, and ammonia loss on peptide N-terminus. Scaffold was used for validation of peptide identifications with confidence filtering of 90% confidence to 99.99%.

2.8 Data Analysis

The folate binding assays and N-homocysteinylation assays were performed in triplicate. The means of these replicates were used for the raw data analysis. The data was analyzed using MANOVA and Linear Mixed Model analysis in SPSS (IBM). In short, data was imported from Microsoft Excel into the SPSS software. The diets and genotypes were assigned bin numbers and were used as the independent variables. The outputs (somite number, folate levels, and N-homocysteinylation levels) were entered as the dependent variables. Default parameters were used for both the MANOVA and Linear Mixed Model analysis.

3. Results

3.1 Mass Spectrometry

The precision and reproduction of the chemical N-homocysteinylation assay was confirmed by mass spectrometry. There were 4 sites of lysine N-homocysteinylation modification detected at 99.99% confidence. In addition, 3 other sites were observed with >90% confidence. Protein identification information about the seven sites is provided in Table 1. The MS/MS data are shown in supplemental Figure 1, along with BSA coverage information at 90% and 99.99% coverage. There were a number of unmodified peptides observed in both samples, so the enrichment is not completely specific in removing N-homocysteinylation peptides from background.

3.2 Adaptation to Dot Blot assay with Known Animal Models

The chemistry and basic application of this N-homocysteinylation assay were developed by the Zhou Laboratory [41]. In this publication, we demonstrate the overall biological relevance and functionality of this assay technique by adapting it to a high throughput format using dot blots. We successfully adapted this assay methodology to examine two mouse models previously shown to exhibit elevated homocysteine levels. Figure 1 shows the

detection of elevated N-homocysteinylation in both tissue from Pcftnull mice compared to Pcft heterozygotes, and in plasma samples from Mthfr heterozygous mice when compared to wildtype mice. The tissue samples used here were from adult age and sex matched mice for assay validation before proceeding to maternal and embryonic samples. The elevations in the N-homocysteinylation levels of the Mthfr and Pcft mouse tissue presented here is similar to the elevations seen in previously reported studies [36].

Experimental Animals and Statistical Analysis—Table 2 shows the number of embryos collected from each genotype and respective diets. During collection, embryos were developmentally staged through somite counting. The embryos were appropriately processed in order to measure folate and N-homocysteinylation levels. Full data tables of raw folate and N-homocysteinylation data for each individual embryo can be seen in supplemental Table 1. N-homocysteinylation and folate levels were also determined in plasma from the dams.

The initial analysis involved a comparison among the three diets with respect to maternal levels of N-homocysteinylation and folate levels, in order to demonstrate that the 1% methionine diet led directly to increasing N-homocysteinylation levels. A two-tailed T-Test confirmed that the high methionine diet produced a statistically significant elevation of plasma N-homocysteinylation (Figure 2a). In addition, the folate levels of the dams were seen to be lower in the high methionine diet and the 2ppm folic acid diet when compared to the 10ppm folate diet (Figure 2a).

Reliability analyses were performed to determine if the embryonic folate and Nhomocysteinylation levels were inversely correlated, as was observed in the adult tissue. The result of an inter-item correlation matrix analysis showed that the correlation coefficient for folate and homocysteine was 0.982, indicating a high degree of inverse correlation between folate and homocysteine throughout all embryos. The positive correlation coefficient is a result of the competitive binding folate assay used, where the results from this assay reflect an inverse of the total folate status of a sample. The use of a MANOVA test showed that both diet and genotype exhibited demonstrable effects on somite numbers, folate levels and N-homocysteinylation levels (Wilk's Lambda Coefficient $p < 0.05$) Figure 2. The average values for each of the embryonic groups can be seen in Supplemental Figure 3. Mixed Model Analysis was used to determine whether correlations, if any, existed between maternal and embryonic homocysteine or folate levels, and also if any correlation existed between maternal folate and embryonic N-homocysteinylation concentrations, somite numbers, or total folate concentrations. We observed no statistically significant correlations between either maternal folate or N-homocysteinylation levels, and that of the embryo with respect to folate or Nhomocysteinylation levels, or even somite numbers. Embryonic N-homocysteinylation and folate levels were observed as dependent upon genotype and the maternal diet, as opposed to directly dependent upon maternal folate and N-homocysteinylation levels

4. Discussion

There is a great need to be able to develop a highly sensitive assay determining N-homocysteinylation levels of proteins that can be adapted to a high throughput format for use with biological samples. The multitude of pathologies that have been correlated to high levels of homocysteine alone merit more intensive investigation into the possible roles that this metabolite may have in causing a variety of disease states. One of the most well accepted hypotheses is that high levels of homocysteine cause more N-homocysteinylation events, which can cause protein malfunctions and plaque formation. Currently, the bulk of the studies relating homocysteine levels with pathologies are correlative in nature. The only way to validate the causal role of N-homocysteinylation is to be able to reliably and quickly determine the levels of this posttranslational modification in cases of NTDs, heart disease, Alzheimer's and more. Herein we described a method for detection of N-homocysteinylation, and its first application, with a large set of mouse embryos that are genetically or metabolically predisposed to NTDs.

Currently used protocols determining levels of N-homocysteinylation include liquid chromatography and mass spectrometry coupling (LC/MS)[49] and antibodies[15],[16]. Although protocols such as LC/MS are highly specific, the nature of the assay limits the number of samples that may be analyzed in a short period of time, with a high-associated cost. The polyclonal antibodies that have been produced have been limited in use because they are not commercially available and apparently have high background rates of non-specific binding, discouraging investigators from utilizing this reagent. In the present study, we have demonstrated the robust adaptability of a chemical method of determining N-homocysteinylation levels [41] to large numbers of biological samples. The benefits of this method include low cost and the stoichiometric reaction, which requires only small amounts of sample on which to perform the assay. Like antibodies, the described reaction may also be tailored to histology samples, such that the location of N-homocysteinylation can be directly visualized in relation to other relevant physical markers. The wide availability of different commercially available colorimetric streptavidin probes would render the demonstration of the location of these modifications very feasible. We also demonstrated the apparent ease and accuracy with which this assay can be modified to adapt to a variety of biological samples. The use of previously studied mammalian models substantiated the ability to adapt this assay to assess total N-homocysteinylation in whole tissue and plasma. Both the *Pcft* null animals and the *Mthfr* heterozygous animals showed significantly elevated levels of N-homocysteinylation, as has already been determined by the use of a radiolabelled high performance liquid chromatography assay[36]. We have shown that the assay described here can also be used to determine relative N-homocysteinylation levels accurately, but without complicated and costly equipment, smaller sample sizes, and with more versatile applications.

Using this methodology, we compared the folate and N-homocysteinylation levels of a cohort of embryos and their dams. The dams were fed three different diets, one high in folate, one control, and one high in methionine. The embryos were one of 4 potential genotypes (*Folr1* $+/+$ and *Lrp6* $+/+$; *Folr1* $+/-$ and *Lrp6* $+/+$; *Folr1* $+/-$ and *Lrp6* $Cd/+$; *Folr1* $+/+$ and *Lrp6* $Cd/+$). Maternal diet and embryonic genotypes were observed to exhibit

significant effects on embryonic folate and N-homocysteine concentrations, as well as on somite number. The test diet was observed to significantly influence the maternal one carbon environment with respect to folate and N-homocysteinylation measurements. It is not surprising that significant changes such as those elicited through the maternal diet would affect the pregnancy outcomes. Interestingly, the embryos appeared to exhibit inverse correlations between folate and Nhomocysteinylation, indicating that the relevant metabolic relationship is established early on in the developing embryo. Such a correlation has been previously observed in tissues from adult rats and mice[50] [51] [52]. The establishment of such a reciprocal relationship between folate and homocysteine so early in development emphasizes the importance of a correct balance of these metabolites, not only for mothers, but in developing embryos as early as the pre-neural tube closure stage. We found no statistically significant correlation between the folate and homocysteine levels in the dams and the embryos, nor in terms of having any impact on embryonic development. The apparent significant influence of both maternal diet and embryonic genotype on embryonic growth and developmental parameters, as measured by somite number, suggests that both maternal diet and embryonic genotype heavily influence pregnancy outcome. This is by no means a new hypothesis[39]; however, we now demonstrate that maternal diet and embryonic genotype are more significant indicators of the course that an early pregnancy may take, rather than markers of embryonic metabolic status, such as degree of N-homocysteinylation. It still must be considered however, that this is the first reported study of N-homocysteinylation levels during early embryonic development. The assay described in this paper provides an easy and high-throughput way to continue the exploration of embryonic and maternal N-homocysteinylation levels during development and even in adulthood.

To our knowledge, studies of maternal and embryonic N-homocysteinylation and folate levels at an early embryonic timepoints have never been previously reported. Recently, Bossenmeyer-Pourie, et al[53], demonstrated that N-homocysteinylation of structural proteins in the stomach of embryonic day 20 mouse fetuses showed an increase in N-homocysteinylation when reviewing animals consuming a methyl donor deficient diet[53]. The embryos that we investigated here were collected and assessed much earlier in development, even before the gastrointestinal structures have formed. Further studies with embryos at and before neural tube closure should be performed emphasizing analysis of N-homocysteinylation of proteins in more specific spatial regions of the embryo. This could be accomplished utilizing histological methods or isolating certain regions/structures of the developing embryo, tasks that will be facilitated by the method explored here. The entirety of the embryo being analyzed also triggers the perceived challenges of so-called “global perspective”. The differences that may be pronounced in a certain tissue type with key developmental proteins, or targets of N-homocysteinylation may be diluted when looking at the background levels that occur in the whole embryo. It should also be noted that the time point observed in our studies was very early in pregnancy. N-homocysteinylation events occur and accumulate over time. We contend that there may be deleterious levels of N-homocysteinylation that have not yet manifested themselves at the times, which we observed. The rapid rate of development and changing metabolic and proteomic profile of embryos at and around neural tube closure necessitates multiple timepoints to be analyzed

during this critical embryonic period. A high throughput assay, such as described here, would make it possible for future exploration into the dynamics of developing embryos. It is also possible that the most important metabolic factors which influence development are more significantly correlated with the mother's dietary status, and are not generally expressed during early embryonic development.

The generation of a data set which begins with the threshold analysis of two relevant and folate responsive genetically modified mouse models may be a precursor to the more complete understanding of the dynamic interaction between genetic forces, environmental influences, and NTD-affected pregnancies. The data presented here emphasize the apparent importance of genotype and diet on embryonic development at the critical period of neural tube closure. The maternal diets and embryonic genotypes significantly affected the metabolic outcomes of the embryos with respect to protein N-homocysteinylation and folate, as well as the developmental outcomes of the embryos, as seen by total somite number. The impact of genetic factors on the development of embryos is obvious here, and has also been extensively studied. The maternal diet; however, along with maternal genetics and metabolic efficiency, is what creates the nutritional milieu in which the embryo develops. The importance of the *in utero* metabolic environment is highlighted by the establishment of a normal ratio of folate and homocysteine at only 9.5 days after conception. This relationship between N-homocysteinylation and folate having been established at such an early time point in mouse development suggests that both of these factors are important for developmental outcomes. Indeed, measurements of two other metabolic intermediates, s-adenosyl methionine and s-adenosyl homocysteine, have shown the importance of a well regulated methionine cycle in early staged embryos for proper neural tube closure [54] [55]. The early establishment of this reciprocal metabolic association between folate and N-homocysteinylation supports the idea that Nhomocysteinylation, along with total available folate, may be one of the important metabolic factors in the etiology of NTDs. Our work presents the first application of a chemical-based N-homocysteinylation assay with a sizeable collection of biological samples. We have adapted a chemical assay developed by Zang et al[41] and applied that assay to multiple biological samples with reproducible results. The extensive ramifications of high throughput studies such as this include being able to examine why high levels of homocysteine may be linked to numerous common disease states. Here, these methods were applied on a large scale and allowed for the examination of the developmental status of cohort of embryos with different genetic and dietary influences.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Proof of Chemical N-Homocysteinylation Assay using known Mouse Models

Dot blot method of N-homocysteinylation assay was tested in both plasma and whole liver tissue from Mthfr and Pcft mice respectively. For each chart, N=5 age and sex matched mice. The asterisks indicate a $p < 0.05$ significance with a two-tailed t-test.

Figure 2. Statistical analysis of dam and embryo outcomes

Panel A shows the dietary manipulation of the dams had the desired significant elevation in N-homocysteinylation levels after a 6 week washout period. A two-tailed ttest showed a $p < 0.05$. Panel B indicates that the embryonic genotype and maternal diet both significantly influenced the embryonic somite number, N-homocysteinylation levels, and folate levels. Panel C shows that there was a not a significant correlation between dam folate and dam N-homocysteinylation and any of the embryonic outcomes.

Table 1

Homocysteinythiolactone Modified Peptides in BSA

The peptides that were found in a BSA sample reacted with homocysteine thiolactone and enriched by an aldehyde solid state to show the confidence of the reaction chemistry. All reacted lysines (indicated by a k) mapped to the surface of the BSA crystal structure and have a >90% confidence.

Sequence	Probability	SEQUENT XCorr	SEQUENT deltaCn	X!Tandem	Modifications	Observed	Actual Mass	Charge
(R)DTHKSEIAHR(F)	94%	2.949 0924	0.2114 2247	2.72 124 65	HCysThiolactone (+117)	437 .95 58	1.31 0.85	3
(R)DTHKSEIAHR(F)	92%	2.627 8703	0.2511 8622	1.25 181 2	HCysThiolactone (+117)	438 .03 32	1.31 1.08	3
(K)KFWGkYL YEIAR(R)	100%	4.054 633	0.3047 8325	4.85 387 2	HCysThiolactone (+117)	846 .73 54	1.69 1.46	2
(K)KFWGkYL YEIAR(R)	100%	4.714 7555	0.3228 3342	4.46 852 1	HCysThiolactone (+117)	846 .78 7	1.69 1.56	2
(R)ALKAWSVAR(L)	98%	1.811 7387	0.0863 0793	1.85 387 2	HCysThiolactone (+117)	1.1 18. 67	1.11 7.66	1
(K)YICDNQDTISSK(L)(E)	100%	3.814 7483	0.2828 4302	6.30 980 4	HCysThiolactone (+117)	873 .21 89	1.74 4.42	2
(K)YICDNQDTISSK(L)(E)	98%	3.157 1016	0.1478 094	3.04 575 75	HCysThiolactone (+117)	873 .30 38	1.74 4.59	2
(K)qNCDFQFEKLGEGYGFQNALIVR(Y)	100%			6.79 588	Ammonia-loss (-17), HCysThiolactone (+117)	1.2 86. 96	2.57 1.91	2
(R)LCVLHEKTPVSEKVT(K)(C)	93%	4.114 283	0.1871 0856	1.48 148 61	HCysThiolactone (+117)	644 .00 59	1.92 9.00	3
(R)LCVLHEKTPVSEKVT(K)(C)	91%	3.754 6747	0.1604 7297	1.22 184 87	HCysThiolactone (+117)	643 .62 96	1.92 7.87	3
(K)ATEEQLKTVMENFVAFVDK(C)	100%	2.541 721	0.2891 9214	9.72 124 7	HCysThiolactone (+117)	1.1 58. 84	2.31 5.67	2

Table 2
Embryo Subjects with Dietary and Genetic Manipulations

Embryos were described in binned groups of genotype and maternal diet. These embryos were further used for statistical analysis. For full table of embryonic and maternal data, see supplemental data.

Number of Embryo Subjects		
		N
Genotype	Folr1 +/-; Lrp6 +/-	71
	Folr1 +/-; Lrp6 +/-	53
	Folr1 +/-; Lrp6 Cd/+	64
	Folr1 +/-; Lrp6 Cd/+	69
Diet	10 ppm Folic Acid	85
	2 ppm Folic Acid	81
	1% L-Methionine; 2ppm Folic Acid	91