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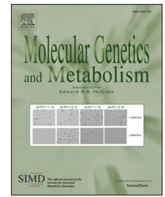

Hyperhomocysteinemia: Related genetic diseases and congenital defects, abnormal DNA methylation and newborn screening issues

Vito Iacobazzi [a,b,c,*], Vittoria Infantino [d], Alessandra Castegna [a], Generoso Andria [e]

[a] Department of Biosciences, Biotechnologies and Biopharmaceutics, University of Bari, via Orabona 4, 70125 Bari, Italy
[b] Center of Excellence in Comparative Genomics, University of Bari, via Orabona 4, 70125 Bari, Italy
[c] CNR Institute of Biomembranes and Bioenergetics, Bari, Italy
[d] Department of Science, University of Basilicata, 85100 Potenza, Italy
[e] Department of Translational Medical Sciences, Section of Pediatrics, Federico II University, Naples, Italy

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ABSTRACT

Homocysteine, a sulfur-containing amino acid derived from the methionine metabolism, is located at the branch point of two pathways of the methionine cycle, *i.e.* remethylation and transsulfuration. Gene abnormalities in the enzymes catalyzing reactions in both pathways lead to hyperhomocysteinemia. Hyperhomocysteinemia is associated with increased risk for congenital disorders, including neural tube closure defects, heart defects, cleft lip/palate, Down syndrome, and multi-system abnormalities in adults. Since hyperhomocysteinemia is known to affect the extent of DNA methylation, it is likely that abnormal DNA methylation during embryogenesis, may be a pathogenic factor for these congenital disorders. In this review we highlight the importance of homocysteinemia by describing the genes encoding for enzymes of homocysteine metabolism relevant to the clinical practice, especially cystathionine-β-synthase and methylenetetrahydrofolate reductase mutations, and the impairment of related metabolites levels. Moreover, a possible correlation between hyperhomocysteine and congenital disorders through the involvement of abnormal DNA methylation during embryogenesis is discussed. Finally, the relevance of present and future diagnostic tools such as tandem mass spectrometry and next generation sequencing in newborn screening is highlighted.

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1. Introduction

Homocysteine (Hcy) is a sulfur-containing, non-proteinogenic amino acid derived from the methionine metabolism. In the homocysteine cycle, Hcy is located at the branch-point of two pathways: remethylation and transsulfuration pathways (Fig. 1) [1,2]. In the remethylation pathway, methionine is resynthesized through two reactions. In the first reaction, which occurs in all tissues, a methyl group is transferred to homocysteine from 5-methyl-tetrahydrofolate (5-methyl-THF) by the vitamin B12-requiring 5-methyl-THF-homocysteine-methyltransferase (folate cycle in all tissues). In the second reaction, which occurs mainly in liver and kidney, the methyl group is transferred from betaine to homocysteine by betaine–homocysteine methyl-transferase (BHMT). In the transsulfuration pathway, homocysteine is irreversibly condensed to serine to give cystathionine by the vitamin B6-dependent enzyme cystathionine β-synthase (CBS). Then, the vitamin B6-dependent enzyme cystathionine γ-lyase (CTH) breaks down cystathionine into cysteine, which could be a precursor of glutathione [3]. In liver, levels of methionine and homocysteine are tightly regulated. Abnormal accumulation of homocysteine results from inability to regulate this pathway and can be attributed to endogenous factors (polymorphisms of the genes coding for the main enzymes involved in homocysteine metabolism) or exogenous factors (dietary deficiency of folate, vitamin B6 or B12) [4]. Increased homocysteine is considered to be an independent risk factor for cardiovascular [5] and cerebrovascular diseases, like stroke or dementia [6], cancer [7], and is highly suspected to increase the risk of congenital defects, such as neural tube defects (NTD), congenital heart defects (CHD), nonsyndromic oral clefts (NOC), and Down syndrome (DS) [8]. However, mild to moderate accumulation of homocysteine due to homocysteine/folate cycle disorders are underdiagnosed.

This review summarizes the most important features related to the hyperhomocysteinemia (hHcy), particularly congenital defects, and highlights the importance of tandem mass spectrometry (MS/MS) and

Abbreviations: AHCY, adenosylhomocysteinase; BHMT, betaine–homocysteine methyl-transferase; Cbl-C, cobalamin C; CHD, congenital heart defects; CTH, cystathionine-γ-lyase; CBS, cystathionine-β-synthase; DS, Down syndrome; hHcy, hyperhomocysteinemia; Hcy, homocysteine; MAT, methionine adenosyltransferase; MS/MS, tandem mass spectroscopy; MTHFR, methylenetetrahydrofolate reductase; NGS, next generation sequencing; NOC, nonsyndromic oral clefts; NTD, neural tube defects; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; THF, tetrahydrofolate; tHcy, total Hcy; WES, whole exome sequencing; WGS, whole genome sequencing.

* Corresponding author at: Department of Biosciences, Biotechnologies and Biopharmaceutics, University of Bari, via Orabona 4, 70125 Bari, Italy. Fax: +39 0805442770.
E-mail address: vito.iacobazzi@uniba.it (V. Iacobazzi).

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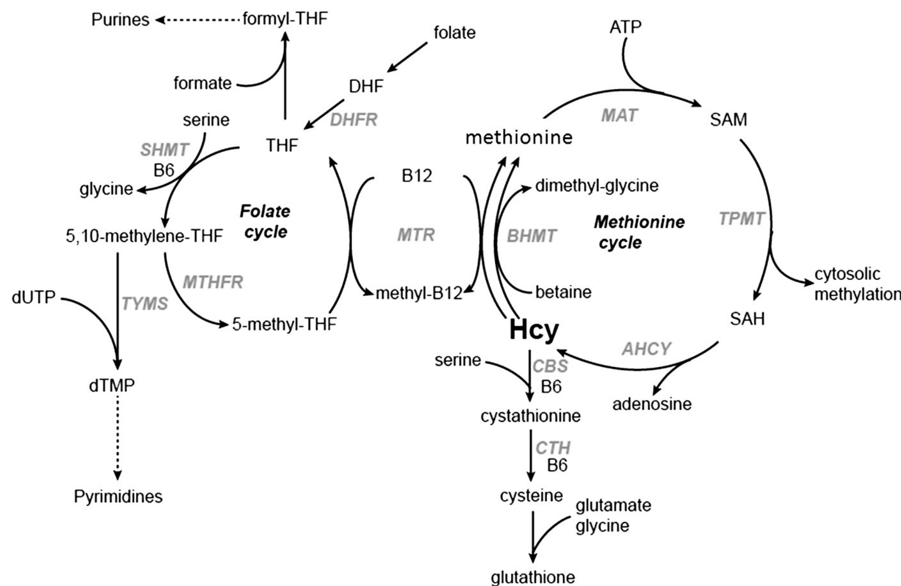

Fig. 1. Schematic representation of the homocysteine metabolism and relationship between folate and methionine cycles. AHCY, adenosylhomocysteinase; B6, vitamin B6; B12, vitamin B12; BHMT, betaine-homocysteinemethyltransferase; CBS, cystathionine-β-synthase; CTH, cystathionine-γ-lyase; DHF, dihydrofolate; DHFR, dihydrofolatereductase; Hcy, homocysteine; MAT, methionine adenosyltransferase; MTHFR, methylenetetrahydrofolate reductase; MTR, methionine synthase; 5-methyl-THF, 5-methyl-tetrahydrofolate; 5,10-methylene THF, 5,10-methylene-tetrahydrofolate; TPMT, thiopurine S-methyltransferase; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; SHMT, serine hydroxymethyltransferase; THF, tetrahydrofolate; TYMS, thymidylatesynthetase.

next generation sequencing (NGS) as fundamental tools for expanded newborn screening.

2. Hyperhomocysteinemia caused by inborn errors of metabolism

The reference values for total Hcy (tHcy) in plasma are 5–13 μM [6]. Circulating homocysteine is present in different forms: 80–90% as protein-bound, 10–20% as homocysteine-cysteine mixed disulfide and homocystine (dimer of homocysteine) and less than 1% as free reduced form [6]. Even though the relative composition of these three fractions of homocysteine can give some information [9], the measurement of total Hcy is considered in the clinical settings of great importance [10]. High Hcy levels are found in the remethylation and transulfuration pathways defetcs that are better clarified by determination of a second metabolite. For example, high Hcy and low methionine indicate a remethylation defect, whereas high Hcy and methionine levels suggest a transulfuration disorder. Evaluation of Hcy levels is also indicative of the folate status, since they are inversely correlated [11]. Epidemiological findings regarding the inverse relationship between Hcy and folate levels also stimulated the interest on Hcy as potentially modifiable risk factor [11]. The main indications for determining tHcy include: diagnosis of homocystinuria, identification of individuals with or at risk of developing folate or cobalamin deficiencies and identification of subjects at risk for cardiovascular disease [12]. Indeed, there is no general agreement on whether plasma homocysteine should be measured as part of any assessment of a patient with vascular occlusive event. It is likely that other measurements, such as B-vitamin, thyroid, and renal functional status, should be evaluated together with Hcy [13].

Several types of hHcy are classified in relation to the total plasma concentrations: moderate (15–30 μmol/L), intermediate (31–100 μmol/L) or severe (>100 μmol/L), respectively [14]. Homocystinuria is a term for a group of disorders characterized by elevated levels of homocysteine in plasma and urine. It is caused by genetic disorders in methionine, homocysteine and transcobalamin metabolism [15]. Dietary deficiencies in folic acid, vitamin B6, and/or vitamin B12 may also cause hHcy [16]. In general, hHcy is observed in approximately 5% of the population [14]. The most severe cases of homocystinuria are due to homozygous (or compound heterozygous) defects in the 5,10-methylenetetrahydrofolate reductase (MTHFR) gene, cystathionine-beta-synthase (CBS) gene and cobalamin (vitamin B12) metabolism genes. Defects in other enzymes of homocysteine cycle cause less severe phenotypes (Table 1). Table 1 reports the mutations found in the genes coding the deficient enzymes and the corresponding ranges of plasma concentrations of methionine, Hcy, SAM and SAH. Often the range of variation is quite large. The extent of elevation depends on the specific mutation(s) causing the impairment of the homocysteine/folate cycle.

2.1. Methylenetetrahydrofolate reductase (MTHFR) deficiency

MTHFR is an important enzyme in the homocysteine metabolism (Fig. 1). So far 67 mutations have been identified in patients with MTHFR deficiency (http://www.hgmd.org). The frequency of mutations is quite common and varies tremendously among different ethnic groups and in different locations. Depending on the mutation, clinical manifestations could be different: acute neurological disturbance in early infancy; progressive encephalopathy in early childhood. However, most mutations are diagnosed in the first months of life. Two functional polymorphisms, C677T and A1298C, respectively, can be associated to a reduced activity. One copy of C677T variant reduces 40% of enzyme activity, whereas two copies result in about 70% decrease of the MTHFR enzyme activity [17]. The variant C677T gives rise to different problems concerning cardiovascular function, homocysteine regulation, DNA regulation, glutathione production, and low methylfolate levels. MTHFR polymorphism A1298C also affects MTHFR enzyme activity,reduces tetrahydrobiopterin levels, and is associated to nitric oxide production leading to aberrant regulation of neurotransmitters [18].

2.2. Cystathionine-β-synthase (CBS) deficiency

Cystathionine-β-synthase (CBS) catalyzes the conversion of homocysteine to cystathionine (Fig. 1). This reaction is the gateway (CBS pathway) for essential biochemical processes, such as glutathione synthesis, a critical component for normal detoxification and defense mechanisms in every cell. The CBS pathway contributes to removal of excess sulfur amino acids. Deficiency of CBS leads to accumulation of homocysteine, methionine, S-adenosylmethionine (SAM), S-adenosylhomocysteine (SAH), and sarcosine with reduced cystathionine and cysteine production (Table 1). Thus, the most effective

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Table 1
Main clinical features, mutations and biochemical characteristics of homocysteine/folate cycle important for different diagnosis.

Enzyme deficiency	Main clinical presentation	Mutations in the deficient enzyme	Met (mM)	Hcy (mM)	SAM (nM)	SAH (nM)	Refs.
CBS	Multi-system abnormalities: musclo-skeletal, eye, nervous system, vascular system	181	353–1891	155–471	888–2.03	147–1.70	[67]
MTHFR	Acute neurological disturbance	67	n.a.	>40[a]	n.a.	n.a.	[68]
Cbl-C	multi-system abnormalities:nervous system, eye, heart	24	n.a.	30–107	n.a.	n.a.	[51]
MAT I/III	In most cases asymptomatic; variable neurological	42 (IA)	206–1394	5.0–26.1	48–120	11–36	[67]
AHCY	Psychomotor delay, myopathy, variable symptoms	10	44–657	2.2–15.4	817–2.971	329–5.04	[69,70]
GNMT	Hepatomegaly (asymptomatic ?)	3	426–1049	7.8–13.0	1.15–3.87	21–72	[71]
Reference range	–	–	13–45	5.5–13.9	92.8 ± 16.2	15–45	[67]

Abbreviations: AHCY, adenosylhomocysteinase; CBS, cystathinine-β-synthase; Cbl-C, cobalamin C; CTH, cystathionine-γ-lyase; GNMT, glycine N-methyltransferase; Hcy, homocysteine; MAT, methionine adenosyltransferase; Met, methionine; MTHFR, methylenetetrahydrofolate reductase; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; n.a.: not available. Range of values are taken from the cited publications.
[a] Mainly from the C677T polymorphism in MTHFR (themolabile enzyme form).

diagnostic parameters arehomocysteine (or homocystine, methionine, SAM, SAH) in plasma or urine. In addition a CBS activity assay in certain cells (*i.e.* cultured skin fibroblasts) can be performed. Infants with CBS deficiency are normal at birth, but, if untreated, will develop the various signs and symptoms associated with the disorder (Table 1). At present 181 mutations have been detected in CBS deficient patients (http://www.hgmd.org).

Patients with CBS deficiency may be classified in two groups: those who respond to therapy with pyridoxine (vitamin B6) and those who do not [19]. Generally, individuals who respond to pyridoxine therapy have a milder form of the disorder, most likely because of residual activity of the CBS enzyme.

Interestingly, some clinical observations [20] indicate that there are subjects with elevated CBS/transsulfuration activity with no apparent CBS mutation. This has probably to be ascribed to individual betaine–homocysteine-methyltransferase (BHMT) down-regulation as consequence of specific mutations. CBS pathway "up-regulations" will result in a higher production of ammonia, urinary sulfates, as well as decreases in glutathione synthesis, and/or possibly imbalances in glutathione redox (reduction/oxidation) ratio.

2.3. Cobalamin (vitamin B12) metabolism defects

The most common inborn error of cobalamin (vitamin B12) metabolism is cobalamin C (Cbl-C) defect that causes an impaired conversion of dietary cobalamin in its two active forms, methycobalamin and adenosylcobalamin, respectively. Methylcobalamin is a cofactor for methionine synthase, required for the conversion of homocysteine to methionine (Fig. 1). Adenosylcobalamin is the cofactor for methylmalonyl-CoA mutase, which converts methylmalonic acid to succinic acid. It is not easy to diagnose Cbl-C defect clinically due to its heterogeneous presentations. Two phenotypes have been recognized: an early onset (within the first year) and a late onset (in childhood or later) presentation [21]. The exact pathophysiology of Cbl-C defects is not clear. Most likely a synergistic effect of different mechanisms, including accumulation of toxic metabolites and deficiencies of metabolites downstream of the defect, are responsible for the multisystem organ involvement (Table 1). An important feature is the toxic accumulation of homocysteine together with SAH that inhibits methyltransferase enzyme resulting in reduction of methionine synthesis, and then SAM. Since this is the principal methyl donor of the cell, SAM unavailability results in inadequate methylation of various biomolecules, such as DNA, histones [22], neurotransmitters [23], proteins and nucleic acids [24].

3. Hyperhomocysteinemia and abnormal DNA methylation: a risk factor in congenital diseases

When Hcy is present in high concentration it competes with SAM for the binding site on DNMT leading to DNA hypomethylation with consequence in epigenetic programming. Evidences from various studies related to different diseases support this hypothesis [25–29]. A genome-wide analysis on human fetal cord blood highlights the possible influence of homocysteine on the fetal epigenome [30,31]. Although the effect of high homocysteine in the developing human fetus has not yet fully established, it is likely that impairment of methylation process during embryogenesis, when DNA methylation is reprogrammed, might play a fundamental role in the etiology of malformations in newborns. Various congenital defects affecting newborns may be associated to abnormal homocysteine and folate metabolism. These defects include: DS, NTD, CHD and NOC.

3.1. Down syndrome (DS)

A general DNA methylation derangement is found in DS subjects resulting in hypermethylation of nuclear DNA [32] and hypomethylation of mitochondrial DNA [33]. Although the molecular mechanism of this alteration is not known, it might be related to imbalanced induction of CBS gene overexpression (located on chromosome 21) that alters homocysteine metabolism leading to compromised folate-dependent resynthesis of methionine [34]. The decreased availability of homocysteine promotes the so called "folate trap", a functional folate deficiency that may contribute to etiology of this complex genetic disorder during embryogenesis [34].

Some studies have shown that mothers with high homocysteine levels together with increased frequency of polymorphism of MTHFR, C677T, are more likely at risk of giving birth to DS affected babies [35]. An elevated risk for DS is also the presence of the MTR 2756G allele, in combination with elevated Hcy concentration [36]. Another study conducted with mothers of children with DS and control mothers analyzed total homocysteine and several variants in the folate pathway (MTHFR C677T, MTHFR A1298C, MTRR A66G, MTR A2756G, and CBS 844ins68) found a correlation between high homocysteine levels and the occurrence of DS in their children, although only the 677T allele was associated with altered levels of total homocysteine. However, when the presence of the alleles was evaluated together, the mothers of children with DS display a tendency to carry a higher number of variant alleles than the control mothers [37]. It has been also demonstrated that Hcy concentrations are higher in DS mother carrying the MTHFR1298CC as compared to the control group [38].

3.2. Neural tube defects (NTD)

Reduction of global DNA methylation might also be a genetic risk for NTD, one of the most common defects at birth. Different studies on mouse embryos support this view. Inactivation of DNA methyltransferase3B has been shown to alter the *de novo* DNA methylation and causes multiple developmental defects including NTD in mice [39]. Inhibition of enzymes of the homocysteine cycle (AHCY and MAT)

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in an *in vitro* chick embryo model shows increased SAH level and reduced SAM/SAH ratio, which correlates to the embryos cranial closure defect [40]. Also proteins located in the neural tube are hypomethylated in cultured rat embryos on low methionine containing media [41].

Similar evidence has been reported also in humans. The SAM/SAH ratio is reduced in mothers of children affected by NTD [42]. Mutation C677T in MTHFR gene, high maternal homocysteine levels and low methionine levels are associated with increased risk of offspring with NTD [43,44]. Global hypomethylation was also found to be associated with NTD in brain tissue [45]. Long Interspersed Elements-1 (LINE1) hypomethylation has been associated with the risk of NTD [46]. Also the specific gene, MGMT, involved in the DNA-repair is hypomethylated in brain tissue of NTD cases [47]. A reduction of DNA methylation was reported in a case with DS and spina bifida [48].

3.3. Congenital heart defects (CHD)

The mechanisms of homocysteine-mediated cardiac defects are poorly understood. Recently, experiments performed on cardiomyocytes have shown that hyperhomocysteinemia related abnormal DNA methylation might be one of the factors for outcome of heart disease. High homocysteine concentrations triggers the epigenetic modification through the activation of NMDAR1 (N-methyl-D-aspartate receptor-1) followed by increased oxidative stress, which leads to decreased level of miR-133 and -499. Decreased level of miRNAs gives rise to an increase of DNMT1 level and likely to an abnormal DNA methylation of different genes. Histone deacetylase (HDCA1) gene results to be hypermethylated leading to a damage of histone deacetylation process. Thus, H3K9 histone acetylation increases resulting in chromatin modification. These changes provide a molecular insight into epigenetic mechanisms that rise in the presence of high homocysteine in cardiomyocytes leading to a cardiac remodeling and dysfunction. Similar results were also obtained *in vivo* on mouse models (CBS +/−) [49]. A meta-analysis demonstrated that maternal hHcy is associated with an increased risk for offspring with CHD. MTHFR polymorphisms (C677T and A1298C) in both mother and children are not independently associated with CHD [50].

Heart defects have been reported to be highly frequent in patients with cbl-C disease [51]. Even though the pathophysiological mechanisms are not well understood, it is very likely that dysregulation of homocysteine cycle and change in expression of some genes by epigenetic mechanism might responsible for the clinical phenotype. Patients with cbl-C defect exhibit a reduced methionine synthase activity, which results in an alteration of homocysteine cycle metabolites levels that may affect cardiac development. It is also likely that disruption of intracellular pool of SAM or change in SAM/SAH ratio may have effect on DNA and histone methylation process [52]. Of note, if perturbation of methylation process occurs during embryogenesis, cardiac development together with other organs might be seriously damaged [53]. Experiments performed on murine embryos carrying methionine synthase [54] and MTHFR [55] knockouts display lethality and developmental defects.

3.4. Nonsyndromic oral clefts (NOC)

Impaired folic acid metabolism and hHcy has been suggested to play a role in clefting. A case-control study showed that mother of children with NOC had higher plasma tHcy concentrations at fasting as well as after methionine loading test, compared to control mothers. Moreover, periconceptional use of folate has been reported to prevent the occurrence of cleft lip and palate [56].

Taken together these data support the idea that embryogenic DNA methylation impairment, maternal hHcy and some methyl cycle gene mutations could be serious risk factors for congenital disorders.

4. Newborn mass screening programs

The true incidences of all homocysteine/folate cycle disorders are only partially known. These abnormalities may remain underdiagnosed for many reasons. First of all, routine screening of these metabolites is not applied to patients with unexplained presentations (especially neurological signs) as the incidence of these genetic diseases is considered extremely low. Furthermore, the panels of metabolites included in neonatal mass screening programs vary in the different countries. In particular, homocystinuria screening is excluded from panels of many laboratories in most nations. Even when countries use the same screening for the same disorders there may be significant differences in practice. Methionine level is the preferred target metabolite included in most newborn screening protocols. Methionine is assayed at birth to identify cases of hypermethioninemia or homocystinuria, which result from deficiencies of MAT and CBS, respectively [57,58]. However, the pyridoxine-unresponsive form of homocystinuria due to CBS deficiency is often undiagnosed since methionine levels might be in the normal range right after birth due to the lower dietary protein intake. As a matter of fact a systematic revision of the literature newborn screening for homocistinuria could not draw any conclusion based on controlled studies, although uncontrolled case-series support the efficacy of newborn screening for homocystinuria and its early treatment. Homocystinuria is better defined and diagnosed when both hypermethioninemia and homocysteinemia are assessed. Quantification of other metabolites of the methyl cycle (methionine, SAM, SAH, Hcy) appears to be fundamental in the clinical setting and diagnosis but this extended panel is not included in newborn screening programs. Therefore, there is an obvious need for improved diagnostic approaches.

4.1. Tandem mass spectrometry (MS/MS)

The need of a better diagnostic approach has been addressed by the development of multitask analytical technologies such as tandem mass spectrometry (MS/MS). MS/MS allows the quantitative simultaneous detection of many metabolites by restricting measurements to specific selected ions and by dramatically increasing the number of disorders screened. Detection of methionine and other methyl-related analytes, such as SAM, SAH and Hcy is now possible in a single run without requesting further sample collection and second-tier assays. For example, it has been proposed to use low methionine as secondary analyte for specific detection of cbl-C disorders among a larger pool of infants with elevated propionylcarnitine (C3) on newborn screening [58]. Often screening centers use the same basic protocols with different cut-offs, with positive predictive values for initial positive test ranging from 4.6% to 81% [59]. Many studies have suggested that lowering the cut-off values produces a beneficial effect in increasing the number of abnormal specimens identified [60], but these efforts did not drive any common policy on this matter. Therefore, adoption of common policies and protocols, management and program evaluation by different laboratories is strongly requested.

Large scale use of a MS/MS expanded screening inevitably raises economic issues as samples positive for a rare disease represent a very small fraction of the analyzed samples due to the extremely low frequencies of the evaluated diseases. This means that the cost associated to a detection is destined to be about 5000-fold higher than that for single MS/MS procedure, assessed at around 7 euros [61]. However, this apparently very high cost is counterbalanced by the fact that it represents the lifetime expense for a patient diagnosed with an inborn error of metabolism, and still much lower than the cost expected for a patient with the same disease but not diagnosed and thus recurrently treated (up to 1 million euros) [61]. Newborn screening by MS/MS thus produces a clear economic advantage.

The real potential of MS/MS screening is still emerging, although the analytical aspects of MS/MS protocols are continuously updated. However, some problems need to be overcome. Most newborn

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screening programs have not reported data on outcome, making difficult to evaluate whether neonatal screening and early treatments before appearance of treatments prevents mortality. Program evaluation by different laboratories is necessary. Management policies, protocols, cut off values are far from being common among laboratories. Efforts to adopt common policies management and follow-up inside a complex and well-organized network linking laboratories with medical units are strongly needed.

4.2. Next generation sequencing (NGS)

While MS/MS screening programs are well established tools of analysis today, recently the possible application of NGS to newborn screening has been proposed and discussed [62]. Pilot projects to examine whether sequencing of newborns' genome can provide useful medical information beyond what current newborn screening already do have been funded by the National Institute of Child health and Development (NICHD) (http://www.nih.gov/news/health/sep2013/nhgri-04.htm). NGS is a high-throughput technology introduced into clinical practice to diagnose single gene disorders as well as diseases caused by a group of related genes and multi-gene disorders. Indeed, the extensive sequencing of the human genome at birth could represent an effective instrument to identify a large majority of Mendelian diseases – including inborn errors of metabolism – and to obtain complete information about late-onset genetic diseases for which effective interventions could be available. Until now, the testing of DNA has not been a first-line newborn screening method, but has been used to confirm the screening results of some disorders, such as cystic fibrosis. A blood specimen collected from the neonate could be tested by whole exome sequencing (WES) or whole genome sequencing (WGS), presumably within current NBS programs. A more practical choice would be that of a targeted exome NGS, limited to a restricted panel of genes encoding the enzymes involved in genetic defects meeting the current criteria for the newborn screening. In addition to identify gene mutations leading to loss of metabolic enzyme activity, array-based analysis of DNA methylation pattern from affected *vs* healthy newborns might reveal new epigenetic mechanisms in the pathogenesis of congenital disease. NGS could legitimately represent an option to be introduced into newborn care, although the clear opportunities are counterbalanced by many challenges, ranging from medical to ethical and socio-economical issues. The main issues to be solved include a lower cost of genomic sequencing, though already rapidly decreasing, a reduced turnaround time for NGS to be set at 2–3 days as required for newborn screening, improvement of bioinformatics tools to provide a clinical interpretation of the data and availability of genetic professionals, trained to communicate the information and formulate management plans.

Together with the previous method, a non-invasive method based on the analysis of circulating cell-free fetal DNA in maternal blood will allow the identification of defects during fetal life [63]. It is known that within the 5th week of gestational age about 10–20% of the total DNA circulating in the maternal blood is cell-free fetal DNA [64]. This DNA is currently used in clinical practice for diagnosis of fetal RhD determination, trisomies 21, 18 and 13 and fetal sex assessment, and to avoid invasive diagnosis in X-linked conditions such hemophilia [65]. Of course, this analysis might be extended to homocysteine metabolism genes and in general applied to all genetic and metabolic defects. Thus, through sophisticated sequencing protocols and bioinformatics algorithm applied to maternal plasma DNA it will be possible to perform a genetic and mutational scans across the whole fetal genome in a non-invasive manner [66]. We can easily foresee that problems similar to those discussed for newborn screening will arise when high-throughput NGS technologies will be applied to non-invasive prenatal molecular testing on fetal DNA for genetic disorders [66], including those of the homocysteine/folate cycle.

5. Conclusion

Considering the important role of homocysteine and folate in the cellular metabolism (protein and DNA methylation, purine synthesis, glutathione production) it is not surprising that homocysteine/folate cycle impairment is associated to a wide range of diseases. It is becoming more and more evident that DNA methylation impairment as consequence of hHcy, caused by endogenous (polymorphisms of the homocysteine/folate cycle genes) and exogenous factors (dietary deficiency of folate), may be significantly involved in the pathogenesis of congenital diseases. Thus, discerning the many factors that may affect the methyl balance and understanding the pathophysiology of these congenital diseases from the “methylation point of view” still remain a great challenge.

MS/MS is a powerful tool to potentially diagnose all these defects. However, according to the current protocols, only classic homocystinuria due to CBS deficiency can be considered a candidate for such screening program, since early detection of the disease can lead to more efficacious treatment. For the other disorders of homocysteine/folate cycle we do not have any evidence of proven efficacy for current therapeutic approaches.

Introduction of microarray, gene-scanning technology and NGS to newborn screening virtually allow analysis and diagnosis of an unlimited number of inherited disorders. In particular, the availability of NGS creates high expectations regarding the potential of this technology in the context of Newborn screening. However, the application of NGS technologies to the diagnosis of genetic disorders, including hyperhomocysteinemias, can be presently considered premature. It is likely that its implementation in newborns will become routine in the (near) future. This possibility will change the landscape of newborn screening, although it will raise new medical, ethical and social problems concerning the consequences of a positive screen test. For example, interpretation of DNA data in a population of healthy newborns is a challenge. In fact, the genotype-phenotype correlation in metabolic conditions often is not straightforward. Storage of genetic information raises other questions, ranging from governance and privacy to handling and accessibility of the data.

In conclusion, NBS programs are aimed to establish a comprehensive system to identify, save, and improve the lives of infants affected by different genetically based conditions. The advent of multitask analytical technology (MS/MS) and, more recently, of sequence testing (WES and WGS) is leading NBS to incorporate new ideas, technologies, ongoing program of evaluation and processes into their systems. Improvements of sensitivity and specificity of screening technologies as well as screening of multiple disorders at the same time, will make future program expansions more practical and help keep cost more manageable.

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