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Review
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S-ADENOSYLHOMOCYSTEINE HYDROLASE (AHCY) DEFICIENCY: A NATURAL MODEL SYSTEM FOR METHYLATION RESEARCH

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Summary

AHCY deficiency is a new human methylation disorder, discovered recently in Croatia, and a natural model for investigating processes related to the methylome. Methylation plays an important role in regulating biological processes and is crucial for gene expression, imprinting, signalling, protein synthesis and lipid metabolism. Thus, methylation has broad impact and provides a suitable base for interdisciplinary research. Linking genomics, proteomics, cellomics, lipidomics and metabolomics and other omics approaches may create a new research avenue – 'AHCYdomics' - a new methylation research platform based on AHCY deficiency. Using such research platform will allow to efficiently explore the full potential of the human methylation disorder AHCY deficiency, and to design methods and approaches that will lead to a better understanding of the human methylome.

Keywords: Methylomics; omics research; methyltransferase; transmethylation; homocysteine; genotype – phenotype

Abbreviations

AHCY: S-adenosylhomocystein hydrolase; SAM: S-adenosylmethionine; snRNA: small nuclear RNA; SAH: S-adenosylhomocysteine; MT: methyltransferase; MS: methinone synthase; BHMT: betaine-homocysteine methyltransferase; MAT: methionine-adenosyltransferase; ATP: adenosine-triphosphate; RBCs: red blood cells; PEMT: phosphatidylethanolamine-methyltransferase; PIMT: protein-isoaspartyl-methyltransferase; PTC: phosphatidylcholine;

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Introduction

At the molecular level, cellular functions form an extremely complex network of interdependent biochemical reactions. Fundamental processes such as cell division, death and differentiation depend on the precise regulation of gene expression, mRNA transcript stability and/or on the posttranslational modifications of effector proteins' functionality.

Methylation of various cellular substrates is directly linked to a variety of biochemical pathways. DNA 5'-cytosine methylation is widely known mechanism of gene regulation, being one of the most important epigenetic mechanisms [1-3] Coordinated methylation of specific arginines and lysines within unstructured N-terminal histone tails creates the 'histone methylation pattern', which is yet another level of fine tuning of gene expression, chromatin stability and epigenetic memory [4-6]. Apart from those epigenetic roles, cellular methylations are also an integral part of different metabolic pathways, including synthesis of monoamine neurotransmitters, detoxification reactions, mRNA, tRNA and snRNA processing [7,8], creatine and membrane phosphatidylcholine synthesis and the repair of proteins damaged by isomerization of aspartyl residues [9]. Lipid methylation might play a role in maintaining membrane fluidity, although still under debate [10,11]; in addition, methylations of specific signal proteins are neccesary for the smooth flow of information within complex signalling cascades [12].

Cellular methylations are catalyzed by substrate-specific methyltransferase enzymes. So far, over 60 distinct mammalian methyltransferases have been described [13], with substrates ranging in size from arsenite to DNA, RNA and proteins. MTs can be classified into 5 different structural families, and some major MT's are shown in table 1; although many of them share similar structural features, more recent studies have proven the existence of extensive functional convergence that is catalyzed by enzymes with remarkably distinct structures [14].

However, all those enzymes share the same methyl donor: S-adenosylmethionine (SAM). After ATP, SAM is the second most abundant enzyme substrate in nature, and the preference for SAM over other methyl donors such as folate can be explained thermodynamically [14,15]. Apart from methylated substrate, SAM-dependent methylation reactions result in formation of S-adenosylhomocysteine (SAH). SAH is hydrolyzed to adenosine and homocysteine by S-adenosylhomocysteine hydrolase (AHCY). While adenosine is further deaminated to inosine by adenosine-deaminase (ADA), homocysteine is either remethylated to methionine or enters the transsulfuration pathway resulting in formation of

cystathionine or, finally, cysteine. Remethylation pathway enzymes methinone synthase (MS) and betaine-homocysteine methyltransferase (BHMT) together with methionine-adenosyltransferases (MATs) and AHCY complete the metabolic cycling of methionine and homocysteine (see figure 1).

Modification	HUGO NC	Methyltransferase (MT) activity
Histone Modifications	SETD7, MLL1-5, SETD1A, SETD1B	H3 K4 Histone-lysine N-MT
	SETDB1, SUV39H1, SUV39H2, EHMT1, EHMT2, PRDM2	H3 K9 Histone-lysine N-MT
	EZH2	H3 K27 Histone-lysine N-MT
	NSD1, SMYD2, SETD2	H3 K36 Histone-lysine N-MT
	DOT1L	H3 K79 Histone-lysine N-MT
	SUV420H1	H4 K20 Histone-lysine N-MT
	CARM1	H3 R2, R17, R26 & H4 R3 Histone-arginine-N-MT
	PRMT5	H3 R8 & H4 R3 Histone-arginine N-MT
DNA modifications	DNMT1	Cytosine-5-MT 1
	DNMT3A	Cytosine-5-MT 3A
	DNMT3B	Cytosine-5-MT 3b
	N6AMT1	Putative N6-DNA-MT
RNA modifications	TRMU	5-methylaminomethyl-2-thiouridylate-MT
	METTL3	N6-adenosine-MT

It is known that SAH strongly inhibits many SAM-dependent methyltransferases [16]. Therefore, it is clear that tight regulation of SAH levels (or the SAM/SAH ratio) is crucial for maintaining the proper methylation potential in the cell. In view of the fact that SAH hydrolysis is the only source of homocysteine in mammals, it is obvious that impaired AHCY activity would affect a wide variety of cellular processes. Knock-out studies in mice have generally confirmed this by demonstrating early embryo lethality in animals harboring deletions in AHCY gene [17]. Elevated homocysteine levels have been reported as a risk factor for dementia and Alzheimer's disease [18] and present a possible risk marker for vascular disease [19, 20].

Three-dimensional structure of human AHCY is solved by X-ray diffraction, and catalytic mechanism has been proposed [21,22]. Reaction steps involve initial oxidation of SAH by the cofactor NAD^+ to the 3-keto derivative, β-elimination

of homocysteine, Michel addition of water and the final reduction step which releases adenosine [23,24]. The reaction is reversible, favoring SAM synthesis, and in this way hydrolytic reaction is dependent on rapid removal of both products. In human, AHCY is a homotetrameric enzyme with a tightly bound single NAD cofactor molecule per subunit [23].

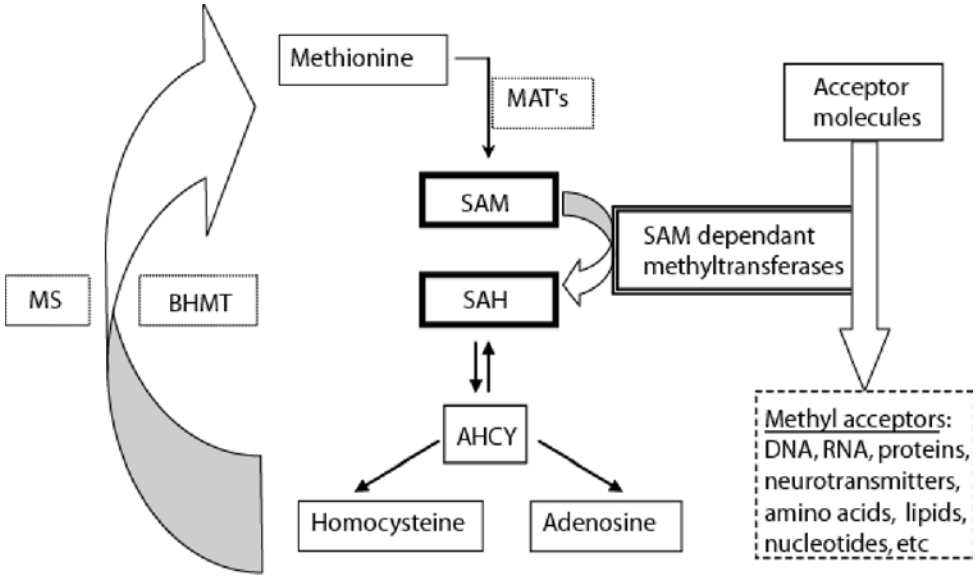

Figure 1. MS, methionine synthase; BHMT, betaine-homocysteine methyltransferase; MAT, methionine adenosyltransferase

Known inherited metabolic disorders of the methionine-homocysteine pathway include MAT deficiency [25,26], GNMT deficiency [27], and the most recently described AHCY deficiency. All three disorders share some common features. In this review, however, we will discuss this novel metabolic disease and some of the advances in understanding its molecular background, structural and catalytic features of mutant AHCY as well as the potential of using the disease as model system for future methylation research.

S-adenosylhomocysteine hydrolase deficiency - Diagnostics and clinical presentation/manifestation

Deficiency of AHCY was firstly described in an croatian infant who exhibited delayed psychomotor development, myopathy and hypotonia, sluggishness,

lack of interest, poor head control and absent tendon reflexes. Physical therapy did not yield in significant improvement of described conditions [28]. Further, until the age of 12.8 months, when the diagnosis was confirmed, the patient's psychomotor development showed very little progress. Also, cognitive functions could not be evaluated due to his brief attention span. Brain MRI showed signs of white-matter atrophy and delayed myelination. Mild chronic hepatitis was detected, and many hepatocytes seemed to lack any rough endoplasmatic reticulum (ER), while smooth ER was hyperplastic. Numerous myelin figures were detected in muscle by electron microscopy, and the authors described the muscle histology as indicative of slowly progressive, destructive myopathy. Biochemical abnormalities included elevation of plasma alanine aminotransferase, aspartate aminotransferase and creatine kinase, prolonged prothrombin time and low fibrinogen. However, it was the levels of sulphur-containing metabolites that set the direction of the diagnostic process. Specifically, the patient showed increased levels of methionine (477-784μM; reference values ranging from 13 to 45μM); SAM was approx. 30x above normal, and SAH was increased over 150x compared to reference values. At the same time, Hcy was only slightly above normal. Such pattern of metabolic deviations has never been described before, and the authors concisely and elegantly ruled out all known disorders that could lead to similar abnormalities, and pinpointed AHCY as the key enzyme in pathogenesis of the disease. Indeed, AHCY activity was significantly decreased as measured in liver extracts and RBC lysates. Sequence analysis of the AHCY gene revealed two mutations: a maternally derived nonsense mutation that introduces a stop codon at amino acid 112 (W112stop) and a paternally derived mutation replacing tyrosine 143 with cysteine (Y143C). The same genotype was also found in two younger brothers of the index patient who also showed very similar symptoms [29, 30]. Information about long-term clinical outcome of AHCY deficiency came from another patient, being the only proven case of the disease in an adult [31]. Only until recently this patient (also showing severe myopathy and developmental delay, accompanied by obsessive behavior and attention deficit disorder) was diagnosed simply with 'isolated persistent hypermethioninemia'. In the light of the discovery of the first AHCY deficiency case, this patient was also tested for SAM, SAH, Hcy and methionine levels, which confirmed AHCY deficiency. Strikingly, although there is no connection to Croatian patients, the same Y143C mutation was found, in addition to alanine to valine exchange at position 89 [32].

The most severe form of AHCY deficiency is presented by an infant patient who died from respiratory insufficiency and liver failure at the age of only 4

months [33]. Metabolic and clinical record raised the possibility that deficiency of AHCY could be the underlying cause of the lethal outcome. By sequencing using the archive material, two point mutations were found, arginine to cysteine exchange at position 49 (R49C) and the substitution of aspartate for glycine at position 86 (D86G) [33].

Genetic background of AHCY deficiency

In humans, AHCY gene is located on the longer arm of chromosome 20 (20q13) and consists of 10 exons. Resulting protein molecule is a homotetramer of 432aa (amino acid) subunits, with one NAD molecule bound tightly per subunit. So far, 5 polymorphic AHCY isoforms were detected in population by starch gel electrophoresis, with some of them characterized at the molecular level [34-37]. None of those polymorphysms seem to have significant effects on the activity of AHCY. Nevertheless, mutations found in patients described above have a great impact on enzymatic capabilities of the enzyme, and are clustered in exons 2, 3 and 4 (figure 2). None of those mutations were found by sequencing AHCY genes from more than 130 control individuals from Croatian population [28], which indicates that Y143C, A89V, D86G or R49C are not common polymorphisms. Both parents of Croatian patients are heterozygous for W112stop and Y143C (mother and father, respectively) and do not show any metabolic abnormalities, although the father's psychomotor development was somewhat slow in early infancy [28]. Accordingly, the trait is characterized as autosomal recessive: despite the homotetrameric structure of the enzyme, the single allele coding for functional AHCY is obviously sufficient (in part, at least) for proper cell function.

The incidence of AHCY deficiency is unknown, not only due to unawareness of the disease but also because of limited availability of specific tests for SAH and SAM [38].

Figure 2. The structure of AHCY gene. Number present DNA base pairs. Coloured boxes present exons.

Contribution of in vitro studies: functional analyses of recombinant AHCY

Effects of the mutations found in AHCY deficient patients on the overall catalytic and physico-chemical properties of the recombinant AHCY enzyme were extensively studied by Vugrek and co-workers. Characterization of recombinant

mutant AHCY revealed the correlation between the degree of inactivation of the enzyme and the severity of clinical manifestation.

Consistent with findings that AHCY activity in WT/W112stop and WT/Y143 heterozygotes (namely, the parents of the first patient) is slightly below normal and that activity in patients' fibroblast extracts is only about 10% of control [28], the authors showed that purified recombinant Y143C AHCY retains only 34% activity as compared to recombinant wild-typ enzyme [39]. As expected, protein truncated by W112stop mutation showed no enzymatic activity. Therefore, it is not likely that the truncated molecule is in fact capable of forming heterotetramers with full-length subunits, since tetramer assembly relies on the C-terminal part of the protein which is completely absent in the truncation mutant. Just as striking were the differences in basic physico-chemical properties of Y143C enzyme: the enzyme was shown to be extremely thermosensitive and prone to aggregation at slightly elevated temperatures, and the bound cofactor accumulated in its reduced (NADH) state. The latter observation suggests that Y143C mutation blocks the enzyme tetramer in its closed conformation. In this view, the catalytic step in which NADH is oxidized back to NAD^+ is impaired. Similar NADH accumulation was observed in human [40] and rat [41] AHCY, also resulting in overall loss of activity. Three-dimensional structure seems to be altered and it will be interesting to examine the crystal structures when they will become available.

The second genotype observed in a patient could be very informative from population genetics point of view, considering that the same mutation (Y143C) was found in a non-related patient in USA. Although the disease was not detected at such early age as was the case with Croatian patients, his overall condition was much more serious. Indeed, the second mutation, A89V, was shown to significantly inactivate the enzyme (>70%), with pronounced effects on three-dimensional structure due to mutation-induced steric clash between amino acids at positions 89 and 84 (Val and Thr, respectively) [32]. The steric incopatibility hypothesis was confirmed by additional *in vitro* mutagenesis experiments. Since mutant A89V AHCY protein is able to form homo-tetramers *in vitro*, it is logical to assume that the formation of Y143C/A89V heterotetramers in patient's cells can explain the decrease in activity of more than 80%, as measured in his erythrocytes [31].

Effects of the last two mutations, found in a deceased infant (D86G and R49C) are clearly related to severity of such outcome. Both mutant proteins showed some unexpected properties *in vitro* [33]. D86G AHCY was almost impossible to express as soluble protein in *E. coli*, refolding has not been succesful, and specific

enzymatic activity could be boosted from initial 1.5% to approx. 15% only by inducing bacterial chaperones. As confirmed by targeted mutagenesis, the existence of negatively charged amino acid at position 86 is crucial for enzyme activity. On the other hand, R49C protein does not even form tetrameric complexes in absence of reducing agents. Authors detected significant NADH accumulation and probable formation of intramolecular disulfides due to newly introduced cysteine residue. Catalytic activity of recombinant R49C AHCY is decreased dramatically, showing only about 7% activity compared to wild-type enzyme. In this way, the results of *in vitro* studies of recombinant proteins clearly reflect the severity of symptoms described in corresponding patients.

It is important to notice that all mutations found in AHCY-deficient patients tend to cluster into exons 2, 3 and 4. Although none of them affect residues that have been directly implicated in catalytic mechanism, it is obvious that they can significantly impair enzymatic capabilities as well as physico-chemical properties of the enzyme. Genotyping of larger number of individuals, combined with standardized SAH and SAM level tests could help estimate the real frequency of AHCY deficiency.

Novel insights into the catalytic mechanism of AHCY

All four mutations found in AHCY deficient patients were studied in detail in our laboratory combining in vitro mutagenesis and in silico modeling techniques [32, 33, 39]. Even though the amino acids directly involved in catalytic mechanism were mostly identified previously [42], we identified some novel primary structure restrictions. Consistent with the fact that it is highly improbable to find missense mutations in AHCY active site in a living organism (especially considering that the enzyme is a tetramer), mutations found *in vivo* have major impact on three-dimensional structure of AHCY, but they do not completely inactivate the enzyme. Rather, they destabilize its structure by disturbing proper H-bond network or inducing sterical clashes leading to changed kinetic parameters.

The AHCY subunit is composed of three domains with the peptide chain organized into 17 α-helices and 15 β-strands [43]. Y143C mutation is located at the end of α-helix 5, which is a part of catalytic domain. In wild-type AHCY a hydrogen bond between Y143 and E115 (α-helix 4) connects both helices, and this connection is abolished in mutated protein with cysteine in place of tyrosine at position 143. In vitro confirmation came from studies of recombinant AHCY where E115L mutation was induced into wild-type enzyme, mimicking the loss of this hydrogen bond as seen in Y143C protein. E115L enzyme showed

properties similar to Y143C AHCY. We proposed the inactivation mechanism in which the open-closed state transitions are impaired by Y143C mutation, blocking the tetramer in its closed state with SAH bound but impairing the efficiency of reduction step of the reaction. This is consistent with the decrease in specific enzymatic activity and the increase in K_m for SAH, as well as with NADH accumulation [39]. A89V mutation inactivated the enzyme by more than 70%. We showed that the reason for this inactivation is the steric clash between Valine 89 and Threonine 84. Although the difference in side-chain volume between alanine and valine is minimal, the position 89 faces the cleft between two domains, which closes upon substrate binding. It was hypothesized that due to the steric clash the substrate or water molecule access/product release was impaired. Additional mutagenesis experiments introducing several amino acids at positions 89 and 84 confirmed this, also emphasizing the importance of polarity of amino acid at position 89 [32]. Regarding R49C and D86G mutations, somewhat less information could be obtained due to high instability of both mutant proteins. Nevertheless, the results indicate that the negative charge at position 86 is important for maintenance of enzyme activity [33].

All amino acids mutated in described studies were implicated in maintaining AHCY activity and stability for the first time. X-ray diffraction experiments will surely reveal much more details; even though these results suggest that the present model of S-Adenosylhomocysteine hydrolysis may need refinement.

Therapeutic approach

All of the patients described above shared similar clinical features: severe myopathy and hypotonia, developmental delay and impaired myelination. Attention deficit/hyperactivity disorder was diagnosed in the first discovered patient; the patient with Y143C/A89V genotype also showed severe obsessive behavior. Apart from characteristic increse in SAM and especially SAH, all of the patients showed high aminotransferases and elevated creatine-kinase [29-31]. The therapy included restricted methionine intake with supplementation of phosphatidylcholine, creatine and cysteine, which is a logical approach considering hypermethioninemia and possible inhibition of MTs, including PEMT. The results must be evaluated considering the patients' age at the beginning of treatment. The oldest Croatian patient started with such therapy at the age of 12.8 months, and showed some gradual improvement of alertness, communicative skills, muscle strength and motor ability. Methionine, SAH and SAM levels decreased, ALT activities were lowered, brain myelination showed remarkable improvement, but CK levels remained high. Motor functions and expressive

speech were affected the most. Both the younger brothers of first patient have been enrolled in theraphy soon after birth, and some improvements were more pronounced (myelination, muscle pathology and liver status). Nevertheless, like the elder brother both remained hypotonic with high CK activities indicating that muscle pathology is not responsive to described therapy [38]. The patient with Y143/A89V genotype has been followed up as a case of isolated hypermethioninemia until recently. Low methionine diet alone did not significantly improve his condition, as expected, considering that he is AHCY deficient; possibly some irreversible damage occurred during long period without proper treatment.

AHCY deficiency and cellular biochemistry

A great diversity of cellular processes is dependent on proper methylation status (or potential) of the cell, and transmethylation reactions have become an area of extensive research. Since enzymes responsible for those reactions generally deploy SAM as a methyl donor and are inhibited by SAH, AHCY deficiency can in fact be considered as the disorder of a cellular 'methylation buffering system'. The questions raised are: which methyltransferases are affected the most, how the degree of this inhibition connects to tissue-specific effects observed, what therapeutic approach would be the most efficient, and what fundamental knowledge can be extracted from those information?

There are probably much more than only 60 enzymes with methyltransferase activity in mammals: 0.6% - 1.6% of all human open reading frames (ORFs) are suspected to code for methyltransferases [44]. The extent of inhibition should vary between those enzymes depending on local concentration o SAH, and the effects should depend on cell/tissue specific role of the enzyme.

Clinical presentation directly points to some candidate methyltransferases. PEMT catalyses the synthesis of phosphatidylcholine (PTC), so the inhibition of this enzyme could cause the PTC decrease detected in patients, which is in turn connected with liver and muscle function. Myelin basic protein-arginine N-methyltransferase is about 90% inhibited by 100x elevation of SAH [45], and myelination insufficiency might be the result of such inhibition. On the other hand, the pathology of the disease is, at least in part, connected to changes in DNA methylation. Repeated measurements by [^{3}H]dCTP incorporation showed hypermethylation of patients DNA, which is opposite of what is expected considering the inhibitory effect of SAH on methyltransferases. The reasons for this phenomenon are still to be elucidated, although it can be speculated that specific DNA MTs are not so sensitive to increased SAH concentrations, and exert

their function properly. Elevated cellular SAM and the fact that some other MTs stay inhibited, therefore leaving excess SAM for DNMTs could explain hypermethylation of patient's DNA. In a exhaustive review by Clarke and Banfield many MTs are listed with their respective sensitivity to SAH [46]: at SAM/SAH ratio of 0.3, DNMT1 retains 23% activity, while PEMT activity is lowered to only 6%. So it can be assumed that strong inhibition of PEMT is the reason for low phosphatydilcholine levels, while the same intracellular SAM/SAH ratios do not have such dramatic effect on DNMTs. This comparison is just an example to show that not all methylations in a cell would be affected to the same extent; further research is necessary at the global genomic/proteomic level. Interestingly, among the enzymes listed in review mentioned, one of the most SAH-sensitive MTs is protein-L-isoaspartyl-methyltransferase (PIMT). This is the key enzyme involved in repair of 'isoaspartyl lesions', namely, isoaspartate residues formed by isomerization of aspartates. Such residues are a measure of non-enzymatic damage that occurs during molecular aging of proteins, so it would be interesting to evaluate this repair mechanism under conditions of low SAM/SAH ratio found in AHCY- deficient patients.

Many of potential disturbances might be cell- or tissue specific, due to differential expression and roles of different enzymes and other macro- and small molecules in various cell types and tissues. On the other hand, some of them are probably more general, like adenosine and glutathione depletion.

The central position of AHCY in methionine pathway and methylation processes indicates that AHCY deficiency affects a wide array of reactions and pathways, thus having the potential to serve as an invaluable model system for both *in vitro* and *in vivo* studies.

AHCY deficiency research – Perspectives and concluding remarks

Methylation of various cellular compounds including DNA, RNA, protein, lipid as well as function of many cellular processes including epigenetic, protein processing, signaling and lipid biosynthesis are affected by AHCY deficiency. Besides that, methylation is important for protein-protein interactions, viral replication, histone modifications, transcription, chromatin structure, X chromosome inactivation, genomic imprinting and chromosome stability. On the other hand, aberrant methylation is a widespread phenomenon in cancer and may be among the earliest changes during oncogenesis.

Study of AHCY deficiency might provide fundamental insights into processes of DNA methylation and other epigenetic modifications. Also, AHCY deficiency is an excellent natural model to study wide range of inherited and

acquired human pathological conditions, in which disturbed methylation is part of the pathogenesis. Thus, activities focusing on methylation and the human methylome should have broad impact.

Figure 3 outlines several avenues for methylome research by using AHCY deficiency as model system. In particular, new insights in the human methylome are envisaged by researching methylation status of:

- Promoter regions of genomic DNA (and other regions, i.e. exons, introns, LTRs, etc)
- miRNA promoters (*one of the hottest topics in research at present moment*)
- mRNA (5′-capping of mRNA etc.)
- a plethora of proteins (Histone lysine and arginine methylation etc.)
- Other molecules and processes mentioned above

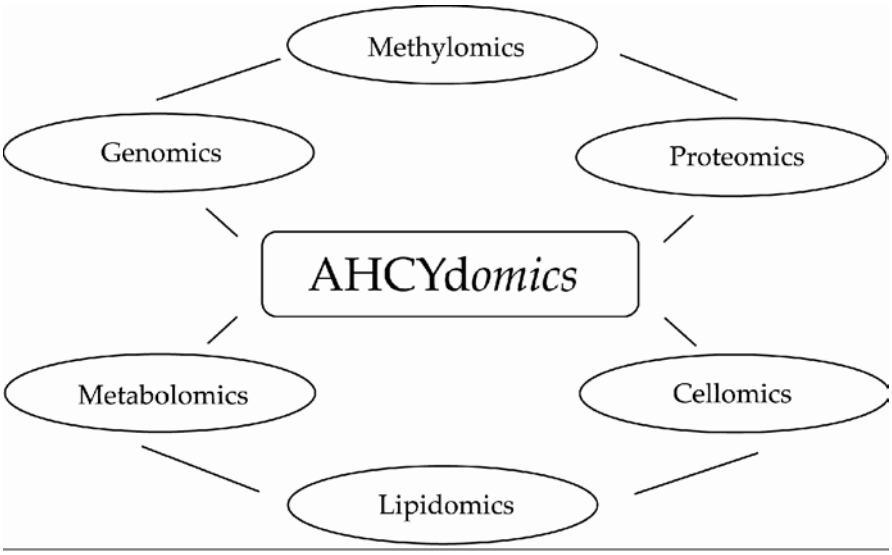

Figure 3: Cartoon showing backbone of research platform AHCYdomics

In summary, using new technology based on 'omics' approaches in combination with AHCY deficiency model system – thereby creating the research platform AHCYdomics, should open up several avenues for methylome research. In particular, research on DNA and histone methylation and other still enigmatic processes controlling gene expression and human development may be tackled using AHCYdomics as research platform.

Acknowledgements

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Sažetak

Nedostatna aktivnost S-adenozilhomocistein hidrolaze (AHCY): prirodni modelni sustav za istraživanje staničnih metilacija

Nedostatna aktivnost AHCY-ja naziv je novog poremećaja metilacijskih procesa kod čovjeka, otkrivenog u Hrvatskoj, koji je prirodni model u istraživanju metiloma. Metilacijski procesi važni su u regulaciji staničnih procesa te imaju ključnu ulogu u regulaciji ekspresije gena, genskog upisa *(imprinting),* prijenosa signala, sintezi proteina te metabolizmu lipida. Upravo zbog tako širokog spektra funkcija, proučavanje metilacije zahtijeva interdisciplinarni pristup istraživanju. Povezivanje genomike, proteomike, celomike, lipidomike i metabolomike te ostalih 'omics' pristupa dovest će do novog integriranog područja istraživanja – 'AHCYdomika' – te stvaranja nove platforme za istraživanje metilacijskih procesa temeljene na nedostatnoj aktivnosti AHCY-ja. Takva istraživačka platforma omogućit će potpuno iskorištavanje mogućnosti koje pruža model baziran na ovom poremećaju, doprinijeti razvoju novih metoda te, nakraju, boljem razumijevanju ljudskog metiloma.

Ključne riječi: methylation disorder; metabolic disease; genotype-phenotype; homocysteine; human mutation; S-Adenosylmethionine (SAM); S-Adenosylmethionine (SAM); S-Adenosylhomocysteine-hydrolase (AHCY)