Nicotinamide *N*-Methyltransferase: Genomic Connection to Disease

David B Ramsden[1] ⓘ, Rosemary H Waring[2], Richard B Parsons[3], David J Barlow[3] and Adrian C Williams[4] ⓘ

[1]Institute of Metabolism and Systems Research, The Medical School, University of Birmingham, Birmingham, UK. [2]School of Biosciences, University of Birmingham, Birmingham, UK. [3]Institute of Pharmaceutical Science, Kings College London, London, UK. [4]Neurology Unit, Queen Elizabeth Hospital Birmingham, Birmingham, UK.

ABSTRACT: Single-nucleotide polymorphisms (SNPs) in and around the nicotinamide *N*-methyltransferase (NNMT) gene are associated with a range of cancers and other diseases and conditions. The data on these associations have been assembled, and their strength discussed. There is no evidence that the presence of either the major or minor base in any SNP affects the expression of nicotinamide *N*-methyltransferase. Nevertheless, suggestions have been put forward that some of these SNPs do affect NNMT expression and thus homocysteine metabolism. An alternative idea involving non-coding messenger RNAs (mRNAs) is suggested as a possible mechanism whereby health is influenced. It is postulated that these long, non-coding NNMT mRNAs may exert deleterious effects by interfering with the expression of other genes. Neither hypothesis, however, has experimental proof, and further work is necessary to elucidate NNMT genetic interactions.

KEYWORDS: NNMT, SNPs, genome, illness, cancer

Introduction

The function of nicotinamide *N*-methyltransferase (NNMT) was originally thought to provide a simple mechanism for regulating nicotinamide (NAM) levels via methylation to the *N*-methylnicotinamide ion (MeNAM) followed by oxidation by aldehyde oxidase and excretion into the urine (Figure 1).

Subsequently, an additional role was proposed – a means of clearing xenobiotics with chemical structures related to that of nicotinamide. This relatively simple picture was challenged by 2 papers published within a year of each other. Elevated levels of the enzyme were described by (1) the group of Hershman, in thyroid cancer[1], and (2) my own group, in the granular layer of the cerebellum in Parkinson disease.[2] Both groups followed up their initial findings with confirmatory papers.[3,4] Subsequent papers reported increased NNMT expression in 14 cancers, including, for example, breast,[5] colorectal,[6] lung,[7] and pancreas.[8] The topic of NNMT and cancer has been reviewed earlier in this journal.[9] Further reports of altered NNMT expression in other non-cancerous illnesses have also been posted. These include heart disease,[10] chronic obstructive pulmonary disease,[11] obesity and type 2 diabetes,[12,13] cirrhosis,[14] and atherosclerosis.[15]

When the first 2 reports were considered, it was concluded that neither the thyroid nor the cerebellum could easily be thought of as major sites of metabolism of nicotinamide or xenobiotics, which posed the question as to what the role of increased NNMT expression might be. In cancer, it appears to be a stimulant to growth, but it is unclear what benefit such a stimulus confers on a cell such as a neuron, which does not replicate. Within non-cancerous tissue, MeNAM anti-inflammatory effects have been reported to influence glycaemic control and lipid metabolism in a tissue-dependent manner,[16] and elevated plasma levels of MeNAM are associated with insulin resistance.[13] In the liver, MeNAM has been designated as a 'good actor' regarding lipid metabolism, whereas in fat cells, it has been labelled as a 'bad actor'.[17] Nevertheless, in a rat model, enhancing the bioavailability of MeNAM has been said to alleviate fatty livers.[18] In brain, MeNAM has been claimed to mitigate diabetes-associated brain disorders.[19]

In addition to the roles of the enzyme and its methylation product, the NNMT gene (*NNMT*) itself has been associated with various illnesses, which poses further questions regarding the role of the gene and its relationship to its transcription products and the NNMT enzyme. Here, we review the literature on single-nucleotide polymorphisms (SNPs) in and near the NNMT gene and their association with disease. To enlighten the discussion, a brief summary of the details of the NNMT gene is given below. The nomenclature and numbering of the gene and its transcription products adopted throughout this review are those given in Ensembl.[20]

Structure of the NNMT Gene (*NNMT*) and Its Messenger RNAs

NNMT occupies 55.5 kb on the long arm of chromosome 11. It possesses 2 major transcription initiation sites (TISs), at positions 11.114257831 (TIS 203) and 11.114295825 (TIS 201),
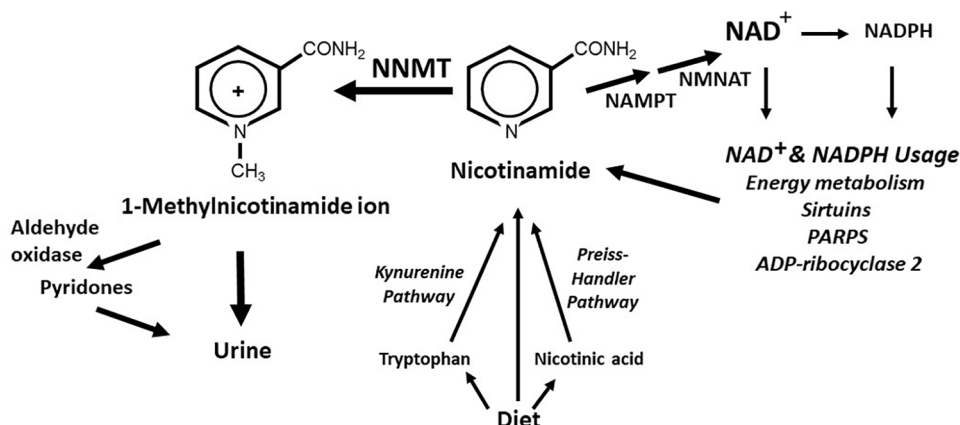
Figure 1. Nicotinamide – from ingestion to excretion.
NAMPT indicates nicotinamide phosphoribosyltransferase; NMNAT, nicotinamide mononucleotide adenylyl transferase; NNMT, nicotinamide *N*-methyltransferase.
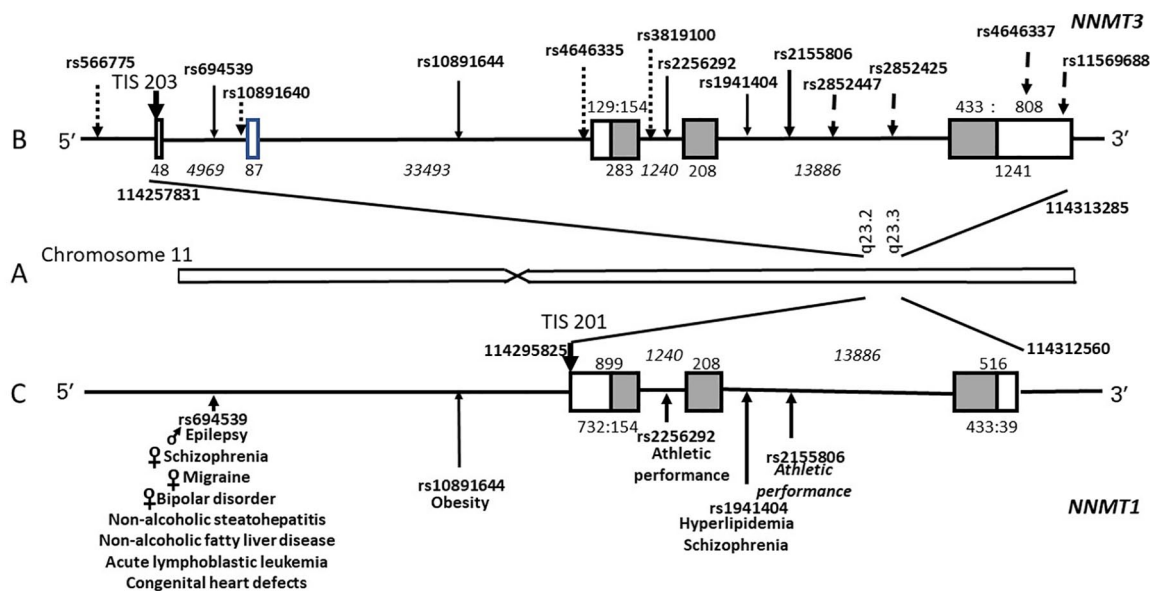

Figure 2. The NNMT gene and its 2 major transcripts.
Solid horizontal lines indicate 5′-flanking region, introns, and 3′-flanking region; white box – untranslated exon, grey box – translated exon; TSI 201 and TSI 203 – transcription start sites of the major transcripts NNMT-201 and NNMT-203. *NNMT1* – solid arrows mark positions of SNPs with significant association with condition indicated. *NNMT3* – dotted arrow marks position of SNPs in haplotype in association with abdominal aortic aneurysm[21]; dashed arrow marks position of SNPs in a haplotype in association with spina bifida.[22] NNMT indicates nicotinamide *N*-methyltransferase; SNPs, single-nucleotide polymorphisms.

respectively. Transcription from TIS 201 primarily gives rise to a transcript of 1710 bases termed NNMT-201 from a gene comprising 3 exons (referred to henceforth as *NNMT1*), all 3 of which possess coding sequences. Transcription from TIS 203 ultimately yields a transcript of 1867 bases including the stop codon (termed NNMT-203) derived from a gene comprising 5 exons (referred to henceforth as *NNMT3*), the first 2 of which are non-coding (Figure 2).

NNMT-201 is by far the most dominant transcript. The gene from which NNMT-203 is transcribed resembles the *NNMT* gene in some lower primates, and so this human gene may be an evolutionary memory within our DNA. Transcription from TIS 201 and TIS 203 yields the same translated product – a protein of 264 amino acid residues, referred to henceforth as NNMT. Five additional, non-coding transcripts of lengths

between 379 and 716 bases arise from a variety of TISs. The function of such transcripts is unclear. Details of all the transcripts are listed in Table 1.

No very detailed description of the mechanism regulating gene transcription has been published to date. Nevertheless, Weinshilboum's group described some features of the 5′ flanking region of *NNMT1* when they published the first description of *NNMT*.[23] Subsequently, several other agents have been described that upregulate NNMT expression in cancer. These include GH,[24] STAT3,[25] c-Jun,[26] TGF-β1,[27] HNF-1β,[3] and androgens.[28] NNMT is also upregulated in non-cancerous diseases such as pulmonary hypertension.[29] Therefore, we undertook the analysis of approximately 2000 bp (base pairs) of genomic DNA upstream of TIS 201 and TIS 203 using the TfSearch[30] and

Table 1. Transcripts derived from the nicotinamide *N*-methyltransferase gene (*NNMT*).

TRANSCRIPT NAME	START POSITION IN CHROMOSOME 11	END POSITION IN CHROMOSOME 11	BIOTYPE	NO. OF EXONS	NO. OF BASES IN FINAL PROCESSED TRANSCRIPT	NO. OF AMINO ACIDS
NNMT-201	114 295 825	114 312 560	PC	3	1610	264
NNMT-202	114 257 787	114 270 517	PT	3	379	—
NNMT-203	114 257 831	114 313 285	PC	5	1867	264
NNMT-204	114 262 888	114 312 208	PT	3	351	—
NNMT-205	114 297 363	114 312 516	PT	2	716	—
NNMT-206	114 298 096	114 312 515	PT	2	511	—
NNMT-207	114 298 151	114 312 515	PT	2	515	—
NNMT-208	114 296 423	114 298 327	RI	0	665	—

Abbreviation: NNMT, nicotinamide *N*-methyltransferase.
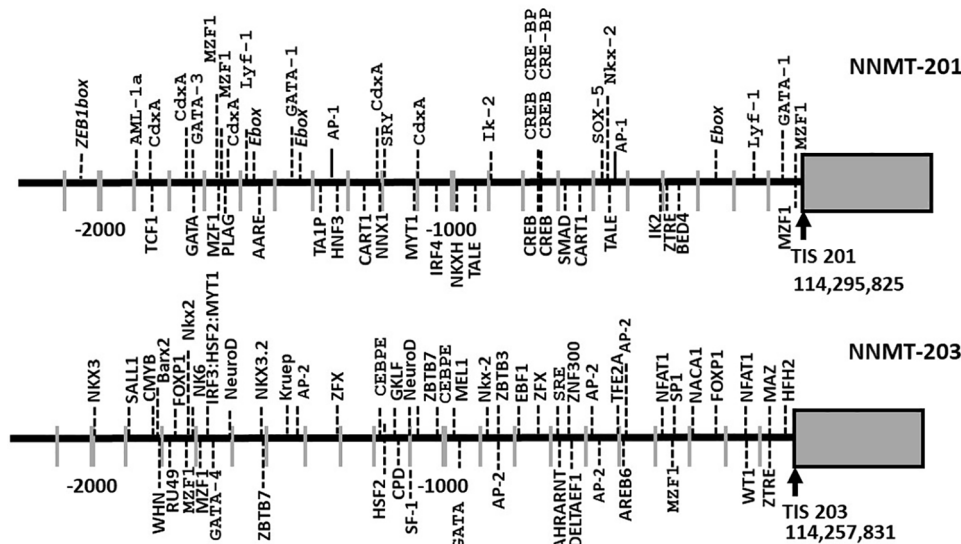
Figure 3. Response elements in 5′ flanking regions.
Solid horizontal line – 5′ flanking sequence; solid grey lines – 100 kb spacing; grey box – first exon. Upper panel – NNMT 201 above the solid horizontal line (5′ flanking sequence): Dashed vertical lines and text above 5′ flanking sequence mark positions of cis-acting factors with greater than 95% similarity to core of canonical site revealed by TfSearch analysis, and solid vertical lines and italic text – AP1 sites revealed by TfSearch analysis with between 90% and 95% similarity to the core canonical site; Zeb1 and E boxes revealed by manual search: below the 5′ flanking sequence: Dashed vertical lines and text below 5′ flanking sequence mark positions of cis-acting factors with greater than 95% similarity to core canonical site revealed by MatInspector analysis. Grey vertical line mark distance from TSI 201. Lower Panel – NNMT-203: Above and below 5′′ flanking sequence: Dashed vertical lines and text mark positions of cis-acting factors with greater than 95% similarity to canonical site revealed by MatInspector analysis. Details of the MatInspector analyses are contained in Supplementary File 1. NNMT indicates nicotinamide *N*-methyltransferase.

MatInspector[31] computer programs. These searches revealed a large number of possible response elements. Ones with a greater than 95% similarity to canonical sequences are shown in Figure 3.

There is no TATA-box in either 5′ flanking sequence.

In the sequence upstream of TIS 201, there is a cluster of cAMP/Ca^{2+}-response element binding protein/CRE-BP DNA-binding protein (CREB/CRE-BP) sites and a possible X Core Promoter Element 1 (XCPE-1) site between 600 and 800 bp from the TIS. XCPE-1 is a weak promoter and acts in concert with sites such as Sp1, but such sites do not appear in the sequence. This promoter is in the region initially described by Weinshilboum's group in 1999, which contained the main regulators of transcription.[23] *NNMT1* is an inducible gene and the CREB/CRE-BP sites may be involved in induction via agents that modulate cAMP levels, such as IGF1. Two possible AP-1 sites are located approximately 0.55 and 1.35 kb from the start site, which could account for c-Jun regulation, although these sites have less than 95% similarity to the canonical sequences. There are numerous potential STAT sites, which could be the ones responsible for STAT3 responsiveness.

Table 2. Somatic mutations in *NNMT* associated with cancers.

TISSUE GROUP	ASSOCIATED CANCER/TUMOUR	NO. OF MUTATIONS	NO. OF INSTANCES OF CANCERS/TUMOURS
Endocrine-related tissue	Breast	2	2
	Cervix	1	1
	Endometrium	5	8
	Ovary	1	1
	Prostate	2	2
	Thyroid	1	1
GI and liver	Large intestine	19	22
	Oesophagus	3	3
	Stomach	4	5
	Upper aerodigestive tract	5	5
	Liver	2	3
	Pancreas	1	3
Urinary system	Kidney	1	1
	Urinary tract	4	4
Other tissues	Autonomic ganglion	1	1
	Bone	1	1
	Central nervous system	2	2
	Haematopoietic and lymphoid tissue	3	4
	Lung	12	15
	NS tumour	4	5
	Skin	28	30
Total	21	102	119

Abbreviation: NNMT, nicotinamide *N*-methyltransferase.

Upstream of TIS 203 are very many potential cis-acting elements with cores of greater than 95% similarity to their canonical sequences as shown in the analysis using MatInspector. These elements are crowded together all along the 2000 bp, in greater numbers than seen in the upstream sequence from TIS 201. Why *NNMT*3 and *NNMT1* are not transcribed equally is not clear from this, although it lacks any potential XCPE-1. It should be remembered that the results presented in these paragraphs are from in silico analyses and need experimental verification for any of the sites mentioned in Figure 3 to be classed as functional elements.

Association of SNPs Within or Near NNMT With Cancer

As DNA sequencing gets cheaper and faster, more and more data about the nature of the human gene are becoming available. This has led to more and more SNPs being identified in the human genome, as illustrated by the work of Saito et al.[32] Presently, the genomes of more than a thousand individuals are known. From these data, it is clear that almost any base may be changed when considered against the base predominantly appearing at any given position in the genome. This is true for *NNMT* as shown by reference to the relevant section of the NNMT entry in Ensembl which lists 12033 variants across the gene.[33] A further illustration of this diversity in the genome comes from the Catalogue of Somatic Mutations in Cancer which lists 101 somatic mutations across the coding sections of *NNMT*, as a consequence changing or deleting 85 of the predominant amino acid residues.[34] These mutations are associated with a range of cancers. Of the cancers listed, skin cancers are most associated with the mutations, although, as a group, cancers of the digestive system are more highly associated
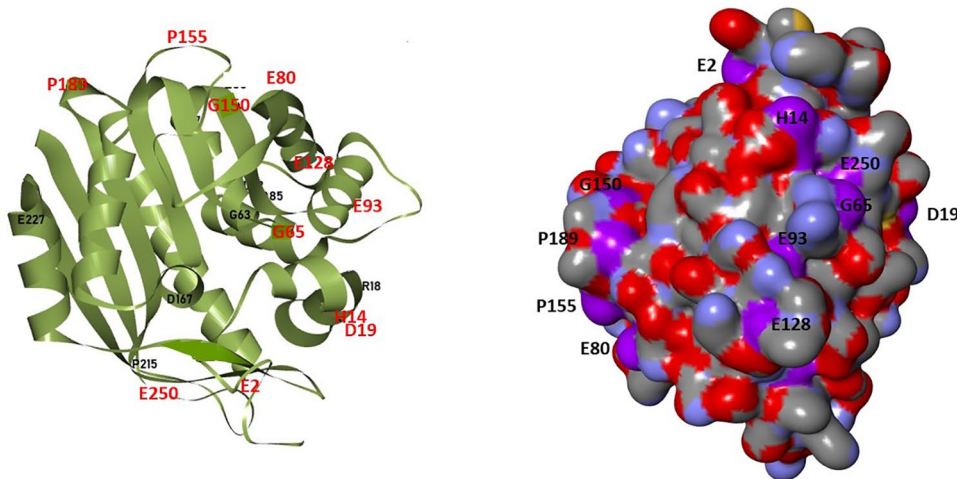
Figure 4. Three-dimensional (3D) views of NNMT showing some mutations associated with cancers of the skin.
Images produced using Discovery Studio Visualizer v4.0 software,[35] atomic coordinates[36]: LH image: spiral – α-helix; tape – β-chain; ribbon – disordered structure; red text – amino acids shown also in RH image, small black text – amino acids that are also visible in this view. RH Image – enzyme depicted using space-filling atomic models of amino acid residues: grey – carbon; red – oxygen; blue – nitrogen; yellow – sulphur. NB: not all the amino acids mutated can be visualised in either view. NNMT indicates nicotinamide *N*-methyltransferase.

(Table 2), in which group the highest incidence is in cancers of the large intestine.

Figure 4 shows 3-dimensional (3D) representations of the NNMT molecule with some of the amino acids mutated in skin cancers.

From this it can be seen that no specific region of the protein has a predominant number of mutations. Rather, mutations appear to be widespread throughout the molecule. The details of the mutations in skin cancers are shown in Table 3.

In addition, in the 21 types of cancer listed in the large majority of cases the mutation is associated with only one instance of cancer, so it is difficult from a statistical viewpoint to claim that these mutations are causal mutations. The highest incidence listed in the Catalogue is 3, and in one of these instances, the same mutation is associated with 2 different cancers, further complicating any claim of causality. Whether this will change as more data are accrued remains to be seen.

One paper which reports on a larger data set is that of de Jong et al.[37] These authors studied SNPs in genes involved in folate metabolism, including *NNMT*, in association with the incidence of acute lymphoblastic leukaemia in 245 patients compared with the frequency of minor genotypes in 500 white blood bank donors in Rotterdam. In one *NNMT* SNP (rs694539; C<T; occurring at position 114 262 697 on the forward strand), the TT genotype had a 2-fold increased risk of acute lymphoblastic leukaemia (odds ratio [OR]: 2.2; 95% confidence interval [CI]: 1.1-4.6; *P* = .04). rs694539 is relatively unusual compared with many of the 12 033 variants across the gene, in that the minor genotype T has a high frequency in normal healthy subject, varying between 17% and 34% depending on the population studied.[38] This point

mutation is located in the introns of transcripts NNMT-202 and NNMT-203, but is very distant from TIS 201 and so the main transcript and the final protein product are unaffected structurally. The SNP affects a number of potential cis-acting transcription factors, but no experimental evidence is available indicating that it has any influence on gene transcription.

SNPs in and Around *NNMT* in Association With Non-cancerous Illnesses
The data in this section are summarised in Table 4.

NNMT and neurologic/psychiatric illness

When illnesses other than cancer are considered, data sets are generally large, allowing statistical significance to be drawn. One particular SNP, the previously mentioned, rs694539, has attracted most attention. This SNP is a G>A mutation on the reverse strand in genomic DNA. rs694539 has been linked to 4 common mental illnesses – bipolar disorder, epilepsy, migraine, and schizophrenia. Details of the associations are summarised in Table 4.

Given the number of cases reported, it is difficult to dismiss as a statistical anomaly the association of this SNP with these illnesses. In most of the instances quoted, no evidence is presented to show that the levels of the enzyme or its messenger RNA (mRNA) are affected. The exception to this is the case of schizophrenia. Bromberg et al[42] found that there was an approximately 30% decrease in NNMT mRNA levels in the brain frontal cortex of patients with schizophrenia at post mortem (results from 13 patients and 13 controls; *P* = .007). In their genomic analysis of a much larger database

Table 3. Mutations associated with skin cancer.

AA MUTATION	COMMENT	BASE MUTATION	REFERENCE
E250K		G748A	COSM3443649
E227E	Silent	G681A	COSM4556171
P215P	Silent		COSM5371703
P215S	Compound Substitution	C642/3TT	COSM4518415
I195I	Silent	C585T	COSM4501309
V194V	Silent	G582A	COSM3443648
P189S		C565T	COSM3443647
D167G		A500G	COSM1704577
P155L		C464T	COSM3443646
G150E		G449A	COSM5901734
E128	Non-sense	G382T	COSM3443645
G119G	Silent	G357A	COSM3443644
E93Q		G277C	COSM6896131
D85G		A254G	COSM3443643
I81I	Silent	C243T	COSM923292
E80K		G238A	COSM3443642
S77S	Silent	C231T	COSM3443641
S77F		C230T	COSM5932820
L72F		C214T	COSM5893574
Q70Q	Silent	G210A	COSM6899129
G65E		G167A	COSM1704576
G63D		G188A	COSM3868470
K47K	Silent	G141A	COSM3443640
D19N		G55A	COSM3868469
R18R	Silent	G54A	COSM6014675
H14N		C40A	COSM5588463
E2K		G4A	COSM139930
M1?	TSC	G3A	COSM5371702

Italics indicate amino acids shown in Figure 4. Red = oxygen, grey = carbon, blue = nitrogen, yellow = sulphur.

(202 patients and at least that many controls) involving 8 SNPs across *NNMT1* and its 5′ flanking region, 2 SNPs – the ubiquitous rs694539 and rs1941404 – were found to be significantly associated with the disease. Furthermore, a haplotype consisting of the bases with lesser frequency at these 2 sites was very strongly associated with the disease, compared with the association of another 41 haplotypes formed from combinations of the SNPs they studied (Table 4). The authors point out that their sample populations for their mRNA study are understandably small. Nevertheless, the combination of enzyme and genotype results makes this a convincing study. They postulate that the effect is somehow linked to homocysteine metabolism. Bromberg et al did not discuss male/female differences.

In a similar study, Wang et al[48] analysed 7 SNPs (rs694539, rs2256292, rs2301128, rs10891645, rs2155806, rs1941398, and rs2604279) in the genomic DNA from 42 (21 male; 21 female) schizophrenic Han Chinese patients and 86 controls (33 male; 53 female). Four of the SNPs were the same as those investigated by Bromberg et al. No association of the disease in male

Table 4.

CONDITION	SNP	BASE CHANGE	POSITION IN CHROMOSOME 11	TOTAL NO. OF CASES	TOTAL NO. OF CONTROLS	GENOTYPES	SIGNIFICANCE χ^2 (P)	ODDS RATIO (95% CI)	REFERENCES
Epilepsy	rs694539	G>A	114262697	215	239	GG, GA, AA	11.64 (.003)		Sazci et al[39]
				78	73	GA	1.677 (.195)	1.295 (0.875-1.915)	Sazci et al[39]
				14	3	AA	8.676 (.003)	5.479, (1.553-19.337)	Sazci et al[39]
Migraine	rs694539	G>A	114262697	433	229	GG, GA, AA	6.076 (.048)		Sazci et al[40]
Migraine (Females)				351	184	GG, GA, AA	0.0565 (.059)		Sazci et al[40]
Migraine (Females)				115	54	GA	0.652 (.419)	1.173 (0.796-1.729)	Sazci et al[40]
Migraine (Females)				16	2	AA	4.474 (.034)	4.346 (0.988-19.112)	Sazci et al[40]
Migraine (Males)				82	45	GG, GA, AA	1.054 (.59)		Sazci et al[40]
Bipolar	rs694539	G>A	114262697	95	201	GG, GA, AA	13.382 (.001)		Sazci et al[41]
Bipolar (Females)				57	113	GG, GA, AA	15.582 (.000)		Sazci et al[41]
Bipolar (Females)				30	28	GA	13.077 (.000)	3.373 (1.721-6.610)	Sazci et al[41]
Bipolar (Females)				2	1	AA	1.505 (.220)	3.373 (1.721-6.610)	Sazci et al[41]
Bipolar (Males)				38	88	2.118	(.347)		Sazci et al[41]
Schizophrenia	rs694539	G>A	114262697	202	202+		(.0036)		Bromberg et al[42]
	rs1941404	A>G	114298316	202	202+		(.013)		Bromberg et al[42]
NASH	rs694539	G>A	114262697	80	183		9.349 (.009)		Sazci et al[43]
						A	3.793 (.051)	1.725 (0.994-2.996)	Sazci et al[43]
NAFLD	rs694539	G>A	114262697	81	80	GG, GA, AA			Hasan et al[44]
				37	28	GA	(.0167)	1.562 (0.828-2.945)	Hasan et al[44]
				27	1	AA	(<.001)	39.5 (5.210-99.469)	Hasan et al[44]

(Continued)

Table 4. (Continued)

CONDITION	SNP	BASE CHANGE	POSITION IN CHROMOSOME 11	TOTAL NO. OF CASES	TOTAL NO. OF CONTROLS	GENOTYPES	SIGNIFICANCE χ^2 (P)	ODDS RATIO (95% CI)	REFERENCES
Hyperlipidaemia	rs1941404	T>C	114298316	349[a]	358[a]	T	]	]	Brett[45]
				391[a]	268[a]	C	](.000)		Brett[45]
				253	263	TC + TT	}	}	Brett[45]
				117	50	CC	}(.000)	}2.432 }(1.574-}3.534)	Brett[45]
Physical endurance	rs2256292	G>C	114297217		G=0.59#	G	]		Li et al[46]
					C=0.41#	C	](.0011)		Li et al[46]
						GG + GC	}		Li et al[46]
						CC	}(.00)		Li et al[46]
Association with plasma homocysteine concentration	rs694539	G>A	114262697		398		(.017)		Della-Morte et al[47]

+indicates at least 202 subjects; original text unclear as to precise number. } and] mark groups compared in an analysis. # genotype ratio.
Abbreviations: NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; NNMT, nicotinamide *N*-methyltransferase; SNP, single-nucleotide polymorphism.
[a]Alleles not individuals.

patients was seen with any of 7 SNPs, whereas rs694539 was highly associated in females ($P=.017$; Table 4). Across the 7 SNPs, only 1 haplotype was strongly associated with the disease (A G G C T C T, in the order of SNPs listed above), again only in females ($P=.0015$), and 1 was on the borderline of significance (G G G C T C T; $P=.05$). These authors also emphasised the link with homocysteine metabolism. The number of patients and controls was smaller than that reported by Bromberg et al, and Wang et al did not analyse any post mortem tissue, so the findings of Bromberg et al concerning mRNA levels await independent confirmation.

Three other common and related mental illnesses which have been investigated are bipolar disorder, migraine, and epilepsy.[39-41] Again, an association between SNP rs694539 and the illness was reported (Table 4). In these 3 disorders, linkage was found with one sex only. In the case of epilepsy, this was with males, whereas in the other 2 disorders, the linkage was with females. This probably reflects the incidence of the disorder in the 2 sexes. In accordance with a theme, the authors suggest that the disease-genomic associations they report are linked to elevated homocysteine levels. The sizes of the groups in the *NNMT* studies are quite large, but it should be pointed out the association of this *NNMT* SNP with migraine did not feature prominently in one huge genomic study.[49] Similar very large-scale genome-wide studies for bipolar disorder and epilepsy did not highlight SNPs in *NNMR* and adjacent regions of chromosome 11.[50,51] It should be remembered that racial differences play a part in susceptibility to conditions such as migraine, as evidenced by the difference between migraine-SNP associations between Han and She Chinese.[52] The difficulty of including all racial groups and balancing racial differences may go some way to explaining the omission in the large studies referred to above. In 3 of the mental illness studies referred to above, the subjects studied were inhabitants of Turkey, and such subjects did not form part of the large study groups.[39-41]

Finally, in this section on neurological disease, one study of factors which are potential indicators of brain ageing was analysed to determine whether these indicators were related to SNPs in the genome. The DNA of 705 stroke-free and dementia-free subjects, who previously had undergone volumetric brain analysis and comprehensive cognitive testing, was analysed using the Affymetrix 100K SNP to give a genome-wide picture of SNPs of each individual. It was postulated that these data may give an insight into an individual's susceptibility to stroke and/or dementia. The SNP data were related to 8 brain parameters: frontal brain volume, parietal brain volume, occipital brain volume, temporal brain volume, hippocampal volume, lateral ventricular volume, temporal horn volume, and white matter hyperintensity volume. Only one SNP in the region of *NNMT* (rs2847476) was found to be statistically significantly related to one of the brain parameters (lateral ventricular volume), $P=3.0 \times 10^{-6}$ generalised estimating equations analysis, and $P=.001$ family-based association tests analysis.[53] In the original text, rs2847476 was said to be in an intronic location, but Ensembl locates the SNP at position 11.114320294, which is slightly more than 7 kb downstream of the 3′ terminus of *NNMT3* and almost 8 kb downstream of the 3′ terminus of *NNMT1*. Other than from its position, it is unclear what the relationship of this A/G SNP is to either of the 2 principal forms of *NNMT*. Although the distances are within the range that sites may function as cis-acting; none is indicated by Ensembl.[54]

NNMT and liver disease

Non-alcoholic steatohepatitis (NASH) and non-alcoholic fatty liver disease (NAFLD) are associated with one *NNMT* SNP, the ubiquitous rs694539. In the case of NASH, the GG genotype was found to be higher protective ($\chi^2 = 3.793$, $P=.051$, OR: 0.580, 95% CI: 0.334-1.006), whereas patients with the AA genotype had increased risk of having the disease ($\chi^2 = 7.748$, $P=.005$, OR: 7.338, 95% CI: 1.448-37.190).[43] As in the case of NASH, the SNP found to be associated with NAFLD was rs694539 (Table 4). Using transient elastography with controlled attenuation parameter assessment as a measure of liver damage, Hasan et al[44] found that the more severe the level of liver damage, the more significant the association with the AA genotype.

NNMT and heart disease

In a study by van Driel et al,[55] of more than 200 children and their parents, no significant association was found between the incidence of congenital heart defects and SNP rs694539. Nevertheless, when environmental factors (maternal peri-conception medicine use and low nicotinamide intake) together with genotypes GA and AA in SNP rs694539 were analysed using multivariable logistic regression models, a different picture emerged. The most extreme risk involved offspring with GA or AA genotypes whose mother also had either GA or AA genotypes, a low nicotinamide intake, and higher use of medicines in the perinatal period. In these circumstances, the risk of congenital heart defects in the child increased almost 9-fold (95% CI: 2.3-33.0, adjusted *P* value = .002, Bonferroni-adjusted *P* value = .006).[55]

A similar initial result (no association with a single SNP) was seen in a large study of patients with familial abdominal aortic aneurysm (423 patients and 423 healthy subjects of comparable age and sex). Giusti et al investigated that SNPs in 17 genes encoding enzymes involved in methionine metabolism included 4 SNPs across *NNMT* (rs566775, rs10891639, rs4646335, and rs3819100; Figure 2). No association of any single *NNMT* SNP was found, although the minor base of

rs3819100 approached significance. Nevertheless, when haplotypes were considered, a combination of the minor bases of the 4 *NNMT* SNPs – TCAC – was significantly associated with the disease.[21]

NNMT and spina bifida

Lu et al investigated 11 SNPs across *NNMT* in the 5′ region, rs505978, rs683271, rs694539, rs11214921, rs10891641, rs2852432}; in exon 1, rs4646335; in intron 2 of *NNMT1*, rs2852447, rs2852425; 3′ region, rs4646337, rs11569688, in 251 cases of spina bifida and 335 controls. As in the case of abdominal aortic aneurysm, the authors found no risk posed by any single SNP, including SNP rs694539, which features so prominently in the various studies mentioned above. Nevertheless, they did find that a haplotype composed of bases – TCAG – of 4 *NNMT* SNPs (rs2852447, rs2852425, rs4646337, and rs11569688) in white non-Hispanic individuals which was claimed to be protective. Unlike van Driel et al,[55] they did not investigate any environmental effects.[22]

NNMT and athletic endurance

In a carefully selected group of athletes (n = 502), selected for being neither extremely fit nor extremely inactive habitually, Li et al investigated 13 *NNMT* SNPs across NNMT, including rs694539, and compared genotype with performance in 1000- and 50-m runs. Only one SNP, rs2256292, was linked to performance in the 1000 m run. This is a G>C SNP at position 114 297 217 on the chromosome, in the first intron of *NNMT1* (Figure 1). Athletes with the CC genotype (n = 92) were linked to a shorter time to complete the race (*P* = .001) compared with that of athletes with the GG and GC genotypes (n = 408). Individuals with this recessive genotype also had a greater relative maximal oxygen uptake (*P* = .001, n = 22 versus n = 96). Significance was maintained even after adjusting for body mass index and regular exercise. One other SNP, rs2155806, in the second intron of *NNMT1*, closely approached significance. Interestingly, there was no association of any genotype with performance in the 50-m run, suggesting that the linkage of rs2256292 was with energy expenditure over a prolonged period. Li et al[46] did not explore any association of performance with a combination of rs2256292 and rs2155806 genotypes.

Interestingly, the relationship between energy expenditure and MeNAM was explored in a recent paper by Strom et al.[56] These authors subjected overweight men to a combination of food deprivation and vigorous exercise (–5000 kcal/d energy balance) for 4 days, causing a highly significant reduction in white cell fat. Serum MeNAM was found to increase, as was muscle NNMT. Cultured myotubes were shown to secrete MeNAM which stimulated lipolysis directly. The authors proposed that MeNAM is a myokine that enhances energy utilisation in response to low energy availability.

NNMT and obesity

The paper by Strom et al links the topics of this paragraph and the one immediately preceding it. In the recent past, much interest has been centred on the role of the NNMT enzyme and its product MeNAM in relation to human obesity. In a detailed summary of the effects of NNMT, the enzyme was termed a good actor in the liver and a bad actor in fat cell by Trammell and Brenner.[17] This was an attempt to reconcile 2 apparently different sets of results by groups of authors writing about the response to a high fat diet.[13,57] This divergence of results still continues. Some authors report a beneficial effect of NAM/NNMT/MeNAM axis in protecting against a high-fat diet,[58] whereas other authors find the reverse.[59] The conclusion of Trammell and Brenner was that the cell type examined was crucial to the result obtained, but in their deliberations only liver and white fat cells were considered.

How NAM/NNMT/MeNAM axis behaves in the whole human body is clearly more complicated, as shown by Strom et al.[56] Although muscle cells may not be high expressors of NNMT individually, muscle mass forms a major part of the body, and so simply interpreting circulating MeNAM levels in terms of white and liver activity may be too simplistic. Whether the subjects investigated start off obese and continue on their habitual diet and exercise regime or begin a corrective regimen influences the interpretation of results. Dietary composition may also play a part. It has been known for decades that the gut biome synthesises NAM and that the composition of the biome is open to change by material ingested.[60] Thus, the amount of NAM entering the circulation may not be simply that in the food ingested but how this food is digested. Another complication in assessing the workings of the NAM/NNMT/MeNAM axis in groups of humans is the fact that the expression of NNMT is genetically controlled, with approximately 25% of the population being high expressors of the enzyme and the remaining 75% form an ill-defined tail,[61] which may be composed of 50% medium expressors and 25% low expressors,[62] possibly complicated by the actions of other gene products. The mechanism of this genetic control and to what extent it affects expression in tissues other than the liver is still unknown.

A yet further complication has arisen when considering obesity, one more germane to the topic of this review. This comes from the study reported by Zhou et al.[63] They analysed 19 SNPs in *NNMT* in the genomic DNA 289 high-body-fat and 494 low-body-fat male Chinese college students who were selected for not being excessively active or excessively inactive. As a percentage of total body weight, a person in the high body fat group was defined as having 19% ⩽ body fat, and someone in the low body fat as 3% ⩽ body fat % ⩽ 13.5%. Body component was measured using bioimpedance measurement with an XSCAN PLUS body composition analyser. Only one SNP (rs10891644) with an allele gene frequency in the low body fat

group of G:T = 0.7:0.3 was significantly different in the high body fat group (G:T = 0.64:0.36; *P* = .029). The genotype distribution was more highly significant (Table 4).

How this SNP relates to NNMT expression awaits elucidation.

NNMT and lipid metabolism

There are numerous pieces of evidence that body fat is linked to MeNAM and activity of the NNMT enzyme.[64-67] Both human and animal adipocytes synthesise NNMT,[68,69] but this relationship to body fat is complex, depending on which cell is the subject of study, which led to the classification of NNMT as both a good and bad actor,[17] as once more evidenced by the findings of Rudolphi et al.[67] In a mouse model of obesity, these authors found that in white fat cells, the relationship between NNMT activity and body weight was only apparent in incipient obesity. However, the relationship of hyperlipidaemia to SNPs in NNMT has been the subject of only one paper. Zhu et al investigated 19 SNPs in DNA from 395 hyperlipidaemic Han Chinese patients and the 316 controls. Their patients had significantly higher levels of circulating triglycerides, low-density lipoprotein cholesterol, and glucose. Both subject groups were predominantly male and the mean age of the healthy controls was significantly higher (approximately 20 years greater). The genetic distribution in only one SNP, rs1941404, was found to be significantly different in the 2 groups after correction of multiple analyses (Table 4), although 3 additional SNPs (rs10891644, rs4646335, and rs1941404) were significant before Bonferroni correction.[70] Whether the failure of these SNPs to maintain significance represents a type 2 statistical error or a true finding remains to be seen.

NNMT – Association With Homocysteine Metabolism and Illness

The above survey of instances where SNPs in *NNMT* are associated with illness poses the question of how these SNPs become so associated. It is possible that they occur by random chance, given the very large number of polymorphisms across the gene, but that seems unlikely. The most common suggestion in the literature is that these polymorphisms are somehow linked to homocysteine metabolism (Figure 5).

The linkage of SNPs rs694539 and rs1941404 and schizophrenia and rs694539 and migraine, bipolar disorder, and epilepsy with elevated levels of homocysteine is largely based on 2 strands of evidence: (1) the 2 phenomena occur in the diseases and (2) there is good evidence that in other circumstances *NNMT* SNPs and levels of homocysteine may be linked.

Regarding the first of these strands of evidence, there is little doubt that in the cases of bipolar disorder,[71] epilepsy,[45,72] and schizophrenia,[73-75] levels of homocysteine and its oxidation product, homocystine, are elevated. This evidence spans many

years and too many references for all to be reviewed here. In the case of migraine, the evidence is somewhat more circumspect[76,77]; however, targeting methylenetetrahydrofolate reductase is regarded as a potential therapeutic option.[78]

The evidence of the link between an *NNMT* SNP and homocysteine levels stems primarily from the work of Souto et al.[79] These authors examined plasma homocysteine concentration and DNA from 398 subjects from 21 extended Spanish families. They conducted a genome-wide analysis covering all 22 autosomes using 363 DNA markers and related homocysteine concentration to individual the DNA markers. A region on chromosome 11 had the highest log of odds score (*P* = .4). *NNMT* was the gene related to homocysteine metabolism in this region. Following this they chose 22 individuals from the high end tail of the homocysteine distribution and 20 from the low end and sequenced the 1.5 kb of DNA from each subject covering the 5′ region of *NNMT1* and the first exon, the coding regions of all 5 exons of *NNMT*, and the 3′ untranslated region of exon 5. These results allowed the genotyping of known and some newly identified SNPs. The net result was the identification of rs694539 as a major determinant of plasma homocysteine concentration. Nevertheless, the heritability of homocysteine concentration is not simple, as Bathum et al point out in the discussion of their results. In their study of genetic and environmental influences on plasma homocysteine concentration, these authors found that the overwhelmingly important genetic influence on homocysteine levels was the methylenetetrahydrofolate reductase gene locus and the influence of this locus diminished with advancing age. This study was on 1206 healthy twins. rs694539 was included in their analysis and was found to have no influence.[80]

In large studies of the association of homocysteine concentration with illness,

1. Loci in genes other than *NNMT* and its associated SNP, rs694539, have been identified as the strong influence on homocysteine levels.[47]
2. Race and sex are major influences too.[81,82]

Thus, from the above it can be seen that despite the detailed work of Souto et al,[79] the relation of SNP rs694539 to homocysteine metabolism is not simple. Given this, the relationship of other SNPs in *NNMT* to this parameter is probably similarly complex.

The evidence of positive relations of NNMT SNPs with illness presented in the above sections comes from relatively small-scale studies and thus has quite limited statistical power compared with major meta-analyses. Furthermore, the subjects come from only one racial group in each study. It is important that attempts be made to replicate these results in other groups, particularly different racial groups. Nevertheless, if one assumes that these results will be replicated, it poses the question of how these polymorphisms could possibly bring
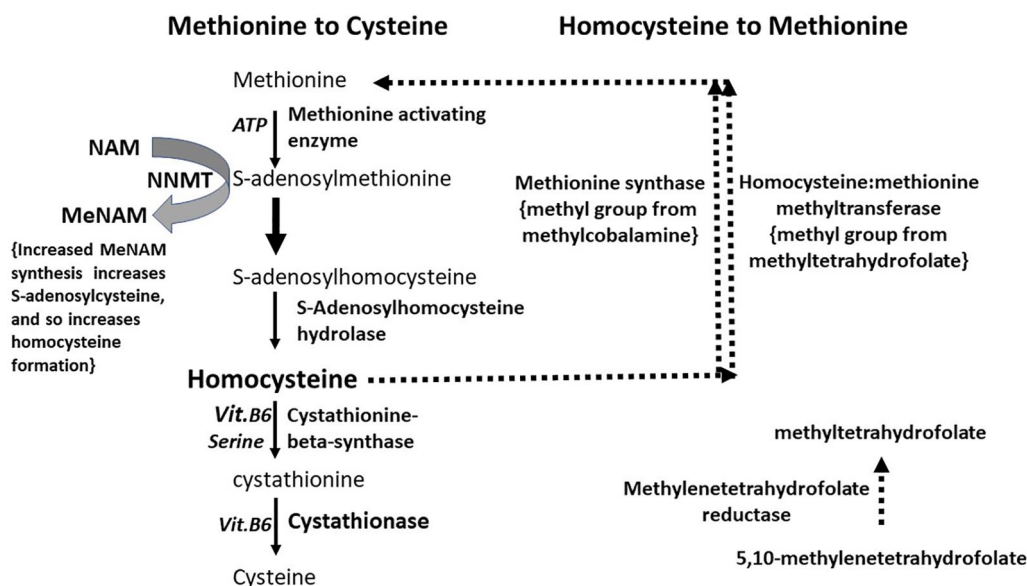
Methionine to Cysteine   **Homocysteine to Methionine**

Figure 5. Role of NNMT in homocysteine formation.
Conversion of nicotinamide (NAM) to *N*-methylnicotinamide (MeNAM) generates *S*-adenosylhomocysteine. NNMT indicates nicotinamide *N*-methyltransferase.

about the susceptibility to the various diseases. Taking the suggestion that homocysteine is somehow involved, elevated levels of NNMT might be expected to consume S-adenosylmethionine (SAM), limiting the availability of methyl groups for the methylation of homocysteine in its return to methionine, thus causing elevated circulating levels of the amino acid. This comes back to the question of how these SNPs might influence NNMT expression if they actually do so. One can see that SNP rs694539 is in a region of the *NNMT* gene that one would expect it to be able to modify gene transcription. The entry in Ensembl indicates that rs694539 might affect the transcription of NNMT-203 and NNMT-202[83] and the website indicates a whole range of possible cis-acting factors that may be changed depending on which of rs694539's 2 bases is present in the DNA. One or more of these cis-acting factors might be capable of affecting NNMT translation. Nevertheless, and importantly for this hypothesis, as yet there is no evidence that SNP rs694539 or any of the other *NNMT* SNPs actually influences transcription. This lack of evidence poses the first difficulty.

The second problem with the homocysteine hypothesis is that in the one instance where NNMT mRNA levels were determined, in schizophrenia, the NNMT mRNA level was found to be decreased not increased. If this decrease in mRNA expression were reflected in NNMT enzyme expression and activity, it would tend to decrease homocysteine formation rather than increase it. It could be that in the post mortem tissue this decrease reflected the effects of the disease over a long period of time and that initially there were increased NNMT levels. Thus, the result at post mortem may not reflect the true state of affairs in the affected tissue. Nevertheless, the hypothesis that *NNMT* SNPs and homocysteine levels are causally linked together via alterations in NNMT, expression in these illnesses remains to be

proved, possibly by showing that in 'healthy' tissue adjacent to the diseased tissue, NNMT mRNA and protein levels are elevated. Without such confirmation, the mechanism involved in the link between elevated levels of homocysteine and the rs694539 polymorphism remains unresolved.

Another consideration to which little attention has been paid is the fact that the expression of NNMT in the liver is strongly genetically controlled. Hepatic expression of NNMT is by far the highest expression in human tissues. Furthermore, hepatic expression appears to be the determining factor of MeNAM excretion in healthy subjects.[84] It is not clear that this pattern of hepatic NNMT expression is true for other tissues, and none of the above studies attempts to relate their findings to hepatic NNMT expression, which complicates the interpretation of their results. It would seem important to clarify whether the level of hepatic expression in healthy subjects relates to any of the SNPs associated with disease, particularly with SNP rs694539.

Having pointed out above the difficulties with the simple homocysteine hypothesis of an SNP affecting NNMT expression which in turn affects homocysteine synthesis, it would be foolish to totally rule out the role that homocysteine formation may play in disease. NNMT is considered as a major determinant of the methionine cycle,[85] and thus is a major player in methyl metabolism, but it may be that indirect pathways are involved. If one assumes that the supply of methyl groups is relatively constant, one may envisage that the fate of methyl groups follows one of the following pathways:

1. DNA methylation;
2. Methylation of nicotinamide by NNMT;
3. Methylation reactions in intermediary metabolism (steroids) or drug metabolism (phenols).

Disturbance of pathway A has the possibility of altering the expression of many proteins, and disturbance of pathway B could alter the energy balance within the cell and/or the balance between NAD^+ and NADPH metabolic pathways. Either of these 2 possibilities may result in ill health, which could increase homocysteine synthesis independently without necessarily influencing NNMT expression.

Assuming that SNPs in and around *NNMT* are associated with disease, which in the cases of schizophrenia and liver disease have been found in independent studies, the question of how these parameters are related is still unclear. Perhaps the single base difference alters either the 3D structure of the genomic DNA or the initial transcription product so that processing of one or other of the transcripts produces an interfering mRNA that disrupts the transcription of one or more genes at sites in the genome, not necessarily *NNMT*. The role of noncoding mRNAs is only gradually becoming elucidated. What is clear is that they influence the transcription, and ultimately translation, of many genes in the aetiologies of different cancers[86,87] and other diseases.[88] As can be seen from Table 1 and Figure 2, depending on the base present, SNP rs694539 changes the sequences of 4 transcripts, and rs2256292 and rs1941404 also alter multiple transcripts. Of the 8 transcripts emanating from the 2 forms of *NNMT*, only 1 transcript (*NNMT-201*) goes on to be translated to any significant extent. Perhaps there is an evolutionary advantage in the formation of these multiple transcripts via the modulation of the transcription of webs of genes. In 3 of the 4 neurological/psychiatric diseases (epilepsy, schizophrenia, and bipolar disorder), altered glucose metabolism, principally via altered mitochondrial metabolism, is thought to play a significant part in disease aetiology.[89-91] In the case of migraine, hypoglycaemia is a trigger in a percentage of subjects.[92] Glucose metabolism is fundamental to physical endurance. NNMT is a regulator of the precursor to NADH, the electron and hydrogen donor for mitochondrial complex I. Perhaps the changes in NNMT in schizophrenia and the relationship of SNPs in and around *NNMT* are related to actions on glucose metabolism rather than on methionine/homocysteine metabolism. The MeNAM itself is capable of increasing ATP production,[93] and it is protective against the toxic effects of homocysteine.[94] Thus, any relationship of the enzyme and SNPs in *NNMT* to homocysteine metabolism, if this indeed the mechanism whereby the SNPs act, is complex. Alternatively, if the mRNAs generated by transcription of *NNMT* is how these SNPs operate, it would suggest that these operations are occurring whichever base is present in any particular SNP, and the effects of such actions are only realised when they predispose to disease.

Conclusions

Reports on a range of diseases, including cancer, neurological, and metabolic diseases, have detailed associations between the disease and SNPs in *NNMT*. In some instances, these associations have not featured in large-scale investigations which have highlighted association with SNPs in other genes. This may suggest that the relatively small-scale investigations that have recorded an association with a particular disease may have only very limited importance. Whether these SNPs in *NNMT* are causal agents in the disease pathways or merely statistical phenomena that occur because of the huge number of genes and SNPs in human DNA remains to be determined. It is also important to note that these associations of *NNMT* appear to be influenced by race and other factors such as age, which emphasises the need for further studies to confirm the universality of these associations. Nevertheless, when these individual reports are taken together, the whole does suggest that the associations do indicate that the NNMT gene is influencing human health, particularly mental health, in ways yet to be determined. The one suggestion of a mechanism, via homocysteine metabolism, does not seem to be valid in the one case where it was investigated. The hypothesis that increased NNMT expression reduces available levels of SAM and so leads to an increased concentration of homocysteine poses the problem that, although homocysteine levels have been found to be increased in schizophrenia, post mortem NNMT mRNA levels were reduced in the region of the brain affected. Generation of different untranslated mRNAs acting on webs of genes, possibly genes involved in glucose metabolism, is suggested as a possible explanation of these genetic associations with disease.

Author Contributions

All authors reviewed and agreed the final text. RHW contributed insight into the relationship of genome to phenotype and the early literature on NNMT. RBP contributed to sections on NNMT expression. DJB contributed the section on NNMT 3D structure. ACW contributed to the section on neurological illness. DBR was the main contributor.

ORCID iDs

David B Ramsden https://orcid.org/0000-0002-0927-1304
Adrian C Williams https://orcid.org/0000-0001-8431-7029

Supplemental Material

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