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SLC19A1 genetic variation leads to altered thiamine diphosphate transport: Implications for the risk of developing Wernicke-Korsakoff's Syndrome

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Abstract

Aims Wernicke-Korsakoff Syndrome (WKS) is commonly associated with chronic alcohol misuse, a condition known to have multiple detrimental effects on thiamine metabolism. This study was conducted to identify genetic variants that may contribute to the development of WKS in individuals with alcohol dependence syndrome through alteration of thiamine transport into cells.

Methods Exome sequencing data from a panel of genes related to alcohol metabolism and thiamine pathways were analysed in a discovery cohort of 29 individuals with WKS to identify possible genetic risk variants associated with its development. Variant frequencies in this discovery cohort were compared with European frequencies in the Genome Aggregation Database browser, and those present at significantly higher frequencies were genotyped in an additional cohort of 87 alcohol-dependent cases with WKS and 197 alcohol-dependent cognitively intact controls.

Results Thirty non-synonymous variants were identified in the discovery cohort and, after filtering, 23 were taken forward and genotyped in the case-control cohort. Of these *SLC19A1:*rs1051266:G was nominally associated with WKS. *SLC19A1* encodes the reduced folate carrier, a major transporter for physiological folate in plasma; rs1051266 is reported to impact folate transport. Thiamine pyrophosphate (TPP) efflux was significantly decreased in HEK293 cells, stably transfected with rs1051266:G, under thiamine deficient conditions when compared with the efflux from cells transfected with rs1051266:A ($p=5.7x10^{-11}$).

Conclusion This study provides evidence for the role of genetic variation, in the *SLC19A1* gene, which may contribute to the development of WKS *in vivo* through modulation of TPP transport in cells.

Short Summary

Genetic alteration in the *SLC19A1* gene is associated with Wernicke-Korsakoff syndrome compared to individuals with alcohol-dependent syndrome with no WKS symptoms. This variation alters the efflux of the thiamine pyrophosphate in an *in vitro* model providing evidence for susceptibility variation which may lead to the onset of WKS.

INTRODUCTION

Wernicke–Korsakoff syndrome (WKS) is the term used to describe the combined presence of Wernicke encephalopathy (WE) and Korsakoff syndrome (KS) (Sechi and Serra, 2007; Zahr, Kaufman, and Harper, 2011). Classically, it is described as comprising of a primary acute neurological phase hallmarked by a triad of ocular motor abnormalities, cerebellar dysfunction, and altered mental state – Wernicke's encephalopathy – followed by a chronic phase characterised by abnormalities in gait and balance, prefrontal neurobehavioral dysfunction, and severe amnesia– Korsakoff's syndrome. **The correlation between the neuropathological findings post-mortem and the clinical features observed during life is generally poor. Thus, only 20% of people diagnosed as having WKS at autopsy displayed the classical triad of clinical features during life, while in ~30% the only salient feature was cognitive impairment (Harper, Giles, and Finlay-Jones, 1986). It follows that WKS is significantly underdiagnosed so that prevalence studies based on clinical findings undoubtedly underestimate the true incidence Current prevalence estimates based on clinical studies vary from 0.04 to 0.13%, while general population rates based on autopsy findings range from 0.8–2.8% (Victor, Adams, and Collins, 1971; Harper *et al.*, 1986).**

WKS is primarily caused by an inadequate supply of thiamine (vitamin B1) to the brain. A very high proportion of affected individuals additionally have a history of chronic alcohol misuse (Kopelman *et al.*, 2009)**. Approximately 30 to 80% of people chronically misusing alcohol are thiamine deficient (Thomson *et al.*, 1987). Data from an unselected post mortem series from Finland reports a WKS prevalence of 12.5% in alcohol misusers (Torvik, Lindboe, and Rogde, 1982). However, in a retrospective review from Australia, only 19% of chronic alcohol misusers with documented neuropathological evidence of WKS at autopsy were diagnosed while alive. Highlighting again that prevalence figures based on clinical manifestations are likely to underestimate the size of the problem (Harper *et al.*, 1986). Proteomic analysis of the dorsolateral prefrontal white matter and the cerebellar vermis in alcohol misusers with no evidence of WKS showed reduced concentrations of the thiamine-dependent enzymes transketolase; pyruvate dehydrogenase E1ß-subunit; and dihydrolipoamide dehydrogenase (Alexander-Kaufman *et al.*, 2006, 2007). However,**

<20% of chronic alcohol misusers develop WKS, raising the possibility that genetic factors are likely to contribute to disease expression. Results from several studies support this hypothesis. Two Japanese brothers, both compound heterozygotes for amino acid substitutions K44E and E320Q in *SLC19A3*, encoding a low-affinity thiamine transporter, developed a syndrome of thiamine-responsive diplopia, ophthalmoplegia and ataxia, similar to Wernicke's encephalopathy, despite having normal serum thiamine concentrations (Kono *et al.*, 2009). Additionally, biochemical differences in transketolase activity have been reported in a set of monozygotic twins, one of whom developed WKS (Leigh, McBurney, and McIlwain, 1981).

Most research interest has focused on the role of the transketolase enzyme in WKS. This was shown, in initial studies, to bind to TPP less avidly in people with WKS than in controls (Blass and Gibson, 1977), but these findings have not been replicated by others (Martin, McCool, and Singleton, 1995; Schenk, Duggleby, and Nixon, 1998). In addition, no differences were found in tissue-specific transketolase isoenzymes, amino acid sequences or differential mRNA splicing in people with WKS compared to controls (McCool *et al.*, 1993; Alexander-Kaufman and Harper, 2009). Other mechanisms, such as post-translational modifications or differential protein assembly, may result in the altered biochemical activity of transketolase in WKS, but thus far there is no evidence for direct involvement of transketolase in the pathogenesis of WKS.

Other genetic loci associated with WKS susceptibility include the Xlinked transketolase-like 1 gene (Coy *et al.*, 1996); the high-affinity thiamine transporter protein gene *SLC19A2* (Guerrini *et al.*, 2005); the γaminobutyric acid A receptor subunit gene cluster on chromosome 5q33 (Loh *et al.*, 1999); and, the aldehyde dehydrogenase 2 gene *ADH2* (Matsushita *et al.*, 2000).

Thiamine is an essential water-soluble vitamin that in its biologically active form, thiamine pyrophosphate (TPP), is an essential coenzyme in several biochemical pathways in the brain. The absorption, transport, cellular uptake, and biotransformation of thiamine are under the control of several enzymes/transporters which are encoded by several genes including *TKT* (Transketolase); *TKTL2* (Transketolase-like 2); *THTPA* (Thiamine triphosphatase); *SLC19A1* (Solute carrier family 19 (folate transporter), member 1); *SLC19A3* (Solute carrier family 19, member 3) and *SLC25A19* (Solute carrier

family 25 member 19) (Figure 1). The possibility that several genetic variants and environmental factors must be present to generate a WKS phenotype, which only becomes clinically relevant when supplies of thiamine are deficient, has not been systematically explored (Sechi and Serra, 2007).

This study aimed to explore possible genetic risk factors for the development of WKS in people chronically misusing alcohol. Exome sequencing data was explored in a discovery cohort of cases with WKS to identify potentially functional variants for validation and, where appropriate, i*n vitro* functional analyses were undertaken.

MATERIALS AND METHODS

Study outline

Exome sequencing data were obtained from 29 alcohol-dependent cases with WKS. These data were filtered to identify potentially functional variants in genes known to play a role in alcohol metabolism, thiamine transport, or encoding for thiamine dependent enzymes. **Putatively functional variants** that were more common in the WKS cases than in European non-Finish population controls were then genotyped in an extended cohort of 87 alcohol-dependent cases with WKS and 197 alcohol-dependent cognitively intact controls. Of the variants genotyped only rs1051266:G in the *SLC19A1* gene was associated with WKS. Stably transfected *SLC19A1* rs1051266:A and *SLC19A1* rs1051266:G cell lines were generated and a series of *in vitro* functional analyses were undertaken under thiamine deficient conditions.

Participants

A UCL cohort of 298 people fulfilling DSM-IV criteria for alcohol dependence was recruited from sites across the UK with the help of the National Institute for Health Research Mental Health Research Network (NIHR MHRN) as previously described (Guerrini *et al.*, 2005). **Here we also make use of data from Mini-Mental State Examinations (MMSE) that had been performed as part of routine clinical practice or where alcohol-related brain damage was suspected.** Participants were classified as *cases* if they fulfilled the ICD10 criteria for the alcohol-induced persisting amnestic disorder (F10.6) and had an MMSE score of ≤26 (Folstein, Folstein, and McHugh, 1975; Wester *et al.*, 2013; Heirene, John, and Roderique-Davies, 2018). Participants were classified as *controls* if they had no history or clinical feature of WKS no evidence of memory loss and an MMSE score of > 26. Genomic DNA was available for all participants. Genomic DNA was also available from 14 deceased WKS cases from the New South Wales Brain Bank in Australia; the DNA was extracted from blood or post-mortem brain tissue, using standard methods.

[FIGURE 1]

Exome sequencing

Available funding supported whole-exome sequencing in 29 cases including 15 from the UCL cohort and 13 from the New South Wales Brain Bank. Exon capture was performed using the TruSeq Exome kit (Illumina, San Diego, CA, USA). Paired-end exome sequencing was performed on a HiSeq2500 sequencer (Illumina) at the UCL Institute of Neurology. **Sequence data was aligned to human genome build GRCh37 with Novoalign (http://www.novocraft.com). Duplicate read removal, format conversion, and indexing were performed using Picard (http://broadinstitute.github.io/picard). Variant calling of the sequence data was performed using the Genome Analysis Toolkit (GATK) (McKenna *et al.*, 2010) separately for single nucleotide variants (SNVs) and insertion-deletion variants (indels). Quality control of the data included filtering of variants such that they had Variant Quality Score Recalibration (VQSR) sensitivities of 99.5% for SNVs and 95% for indels, homozygous and heterozygous genotype quality (GQ) scores ≥ 20 and ≥ 40 respectively; sample read depths ≥ 10, and Hardy-Weinberg equilibrium (HWE) with P >10^{-20}.**

Analysis of the final data was restricted to genes that play a role in alcohol dependence, alcohol metabolism, dementia, thiamine transport, the citric acid cycle and genes that encode thiamine dependent enzymes (Figure 1 and Table 1). The sequence data from these genes were further filtered to identify putatively functional variants which (i) were predicted, based on their Polyphen2 (Adzhubei *et al.*, 2010) and SIFT (Ng and Henikoff, 2003) scores to have an impact on gene function; and/or (ii) had an allele frequency difference of >10% in the European, non-Finnish population in the Genome Aggregation Database (gnomAD version 2.1) (Lek *et al.*, 2016). Two additional variants were detected in the exome sequence data with known functional effects, rs1229984 (Edenberg, 2007) in ADH1B and rs1051266 SLC19A1 (Yee *et al.*, 2010) were also selected. These variants underwent follow-up genotyping in the extended UCL case-control cohort.

Genotyping of WKS cases and controls

The SNPs of interest, identified following exome sequencing, were genotyped in-house in the remaining 87 cases and 197 controls on the K-Biosciences Competitive Allele-Specific PCR (LGC Genomics, Hoddesdon, UK) as previously described (Way *et al.*, 2015).

Statistical Analyses

Statistical analyses were performed using PLINK 1.9 (http://pngu.mgh.harvard.edu/purcell/plink/) (Purcell *et al.*, 2007). The association between each genetic variant and WKS was estimated from logistic regression analysis, and adjusted for sex, assuming an additive genetic model. These analyses were performed in the independent case-control sample to be able to report an independent assessment of association with WKS. False discovery rate adjustment (FDR) for multiple testing was performed with the statistical software program R (version 3.4.4) (R, 2014). An uncorrected cut-off significance value of $p< 0.05$ was used **to identify variants for functional analyses**.

Generation of a stably transfected *SLC19A1*:rs1051266 cell line

HEK293 cells were maintained in Dulbecco's modified eagle medium (DMEM) (Sigma-Aldrich, Poole, UK) supplemented with 10% foetal bovine serum (FBS) (Invitrogen, Life Technologies Ltd, UK) and 1% penicillin/streptomycin (100 U/ml, Sigma-Aldrich). Cells were maintained at 37˚C with 95% O_2 with 5% CO_2. A Myc-DDK-tagged plasmid containing cDNA from the *SLC19A1*-member 1, transcript variant 1, was obtained from OriGene (Cat# RC222669, Origene, Rockville, MD, US). The New England Biolabs BaseChanger programme (http://nebasechanger.neb.com/) was used to design primers to introduce the rs1051266:G allele (WKS associated) into the *SLC19A1*:rs1051266:A plasmid using the Q5® Site-Directed Mutagenesis Kit as per the manufacturer's protocol (New England Biolabs, Hitchin, UK).

Cellular Transfection

HEK293 cells were transfected with the *SLC19A1*:rs1051266:A and *SLC19A1*:rs1051266:G plasmids with Lipofectamine2000 (Thermo Fisher Scientific, Paisley, UK) as per the manufacturer's guidelines.

Stably transfected HEK293 cells containing *SLC19A1*- rs1051266:G and *SLC19A1*-rs1051266:A plasmids were generated by selection of transfected cells using the aminoglycoside G418 (Sigma-Aldrich). A cell kill curve showed that the optimal concentration of G418 in this experiment was 0.8mg/ml (data not shown). The generation of the wild-type and disease-associated cell lines was undertaken following the Lonza technical reference guide for Generation of Stable Cell Lines (https://knowledge.lonza.com/downloadasset.ashx?assetId=28243).

Development of a thiamine deficient *in vitro* model

At concentrations of between 5 and 50mM, thiamine passively diffuses into cells, while carrier-mediated transport occurs at concentrations ≤2mM (Hoyumpa *et al.*, 1982). To determine if the WKS associated variant rs1051266:G alters thiamine transport in the stably transfected cells, it was necessary to develop conditions in which thiamine uptake occurred *via* the SLC19A2 and SLC19A3 transporters, rather than by passive diffusion(Hoyumpa *et al.*, 1982; Rindi and Laforenza, 2000). The average thiamine concentration in normal DMEM, supplemented with 10% FBS, determined using HPLC (method below) is 11.07mM. The lowest medium concentrations of FBS and thiamine needed to ensure HEK293 cell growth, while still maintaining a thiamine deficient environment, were determined in cells plated at a density of $1x10^4$ cells/well in 96-well plates (µ-Plate 96 Well, Thistle Scientific, UK) in (i) custom made thiamine free media (Sigma-Aldrich, UK), (ii) medium containing thiamine in concentrations ranging from (0.05nm-to 5nm) (Sigma-Aldrich, UK) and FBS ranging from 0.2% to 2.2% to a final concentration of thiamine in FBS of 0.22 mM to 2.4 mM.

The numbers of live and dead cells at 24-, 48- and 72-hours after plating under varying FBS/thiamine deficient conditions were quantified using live-cell imaging with the ImageXpress® Micro XL System. Cells were incubated for 15 min with the Calcein AM (1:100, Cat# 1430 Thermo Fisher, UK) which fluoresces when hydrolysed by intracellular esterases in live cells, propidium iodide (1:1000, Cat# P13004MP, Thermo Fisher), which fluoresces in the DNA of dead cells, and Hoescht-33342 (1:1000) a nuclear marker. Cell counts represent the mean from three experiments **(Table S1)**.

Incubation of the cells in the absence of FBS for 24-, 48- and 72-hours led to a 48% reduction in the number of live cells after 24 hours, across all thiamine concentrations, when compared to 2.2% FBS. More substantial reductions in cell numbers were observed after 48 hours (88%) and 72 hours (88%) in the cells collected at these time points. This reduction in cell numbers in the absence of FBS could be reversed by adding 0.4% FBS at 24 hours but there was still a 54% reduction in viable cells at 48 hours and a 55% reduction at 72 hours irrespective of the thiamine concentration used (Table S1). Use of 0.2% FBS at 24 hours appeared to be necessary for cell viability. Based on these results, thiamine deficient culture conditions were defined as 0.2% FBS in thiamine free DMEM and a normal thiamine environment was defined as 2.2% FBS in thiamine-free DMEM.

Comparisons were made between the concentration of thiamine and TPP in the cell culture media of the stably transfected cell lines expressing the rs1051266:A allele compared to the WKS associated rs1051266:G allele, under both thiamine replete and thiamine deplete conditions.

High-Performance Liquid Chromatography

TPP and thiamine concentrations were measured in media samples using an Agilent 1200 series HPLC system. The methods and reagents required for sample preparation were obtained from a "Vitamin B_1 in Whole Blood" HPLC detection kit by Chromsystems Instruments & Chemicals GmbH (Am Haag, Gräfelfing, Germany). Two hundred microlitres media samples or standards were added to 100µl extraction buffer mixed briefly by vortexing; 300µl of precipitation reagent was added, mixed by vortex for 30s and centrifuged for 5 min at 9,000 x g. After centrifugation, 100µl of the resulting supernatant was added to 200µl derivatisation mix in a new reaction vial and mixed briefly; 100µl each of neutralisation and stabilisation solution was added, mixed briefly, and allowed to stand at room temperature for 20 min. Fifty µl of the prepared sample was injected into the HPLC using an isocratic gradient with a flow rate of 1 ml/min. TPP and thiamine were detected using a post-column fluorescence HPLC module at room temperature using the following wavelengths; excitation=367nm and emission=435nm. A calibration curve was produced using the AUC for each standard and plotted against concentration; the resulting slope value was then used to calculate the concentration in the

samples. The mean of three independent biological replicates was taken and differences were calculated using a 2-tailed Student T-test assuming equal variance.

RESULTS

Sample Demographics

Cases and controls were 79% male and 73% male, respectively. Duration of alcohol misuse was longer among the cases compared to the controls (mean number of years 29.92, Inter Quartile Range (IQR) 23-38 in cases: and mean number of years 17.26, IQR 8-26 in controls; t-test $p=2.05x10^{-07}$).

The mean age of onset of alcohol dependence was lower in the cases compared to controls but this difference was not statistically significant (mean age 23.36, IQR 16-30 in cases; and mean age 27.27, IQR 18-33 in controls, t-test p>0.05).

Exome sequencing

Whole exome sequencing of DNA from the 29 WKS cases identified 30 missense variants in a panel of genes involved in alcohol metabolism, thiamine metabolism and/or transport. After filtering, 23 variants were taken forward for genotyping in 87 additional cases and 197 controls (Table 2). A null variant in rs150570593 in *TKTL2* was found in a single post-mortem sample from the NSW brain bank. A further non-synonymous variant, rs1051266 in *SLC19A1* leading to an amino acid substitution of histidine residue 27 to arginine, showed nominal levels of association with WKS (p= 0.010) which did not survive FDR when corrected for all the variants tested, or for all the variants in the thiamine system. These two variants were further explored:

1. The null variant in *TKTL2* (rs150570593), occurs at a frequency of 0.000077 in the Exome Variant Server (EVS; https://evs.gs.washington.edu/EVS/) dataset and a frequency of 0.000117 in the gnomAD dataset (v2.1.1). However, this variant was not identified in any further cases or controls. Thus, it was not further pursued.

2. *SLC19A1* controls the transport of folate into cells and facilitates the export of TPP out of cells (Zhao *et al.*, 2001). The function of the rs1051266 variant on transport uptake has been widely studied as has its association with drug responses and toxicity, particularly methotrexate, a

folate antagonist used to treat malignancies and inflammatory conditions. Methotrexate uptake efficacy and hence therapeutic response is increased in carriers of the alternative A allele (Baslund, Gregers, and Nielsen, 2008). Thus, the effects of this polymorphism in *SLC19A1* was further explored even though the association did not survive correction for multiple testing because of the clear importance of its documented role in therapeutic drug responses.

Effect of *SLC19A1*:rs1051266 on the transport of TPP in stably transfected HEK293 cells

When the transfected HEK293 cells were incubated in 0% FBS the mean (±SD) TPP concentration in the *SLC19A1:*rs1051266:A media (31.43 ± 4.66 nM) was significantly higher than in the WKS associated *SLC19A1:*rs1051266:G media (14.75 ± 0.77 nM) at all thiamine concentrations ($p = 5.7x10^{-11}$) (Table 3). Likewise, when the cells were incubated in 0.2% FBS the mean TPP concentrations were significantly higher in media from cells expressing the *SLC19A1:*rs1051266:A allele than in media from the cells expressing *SLC19A1:*rs1051266:G, again at all thiamine concentrations (20.26 ± 4.27nm cf 38.22 ± 10.46 $p = 2.9x10^{-4}$) (Table 3).

DISCUSSION

In the present study exome sequencing data obtained from 29 cases with well-documented alcohol related WKS were used to identify associations with potentially functional variants in genes involved in alcohol metabolism, thiamine metabolism and/or transport. The key finding was an association at nominal significance between *SLC19A1*:rs1051266:G and the presence of WKS. This gene and its protein product are of considerable interest and were further explored. The efflux of TPP from HEK293 cells stably transfected to over express *SLC19A1*:rs1051266:G under thiamine deficient conditions, was significantly lower than from cells over expressing *SLC19A1*:rs1051266:A

SLC19A1 encodes SLC19A1 (reduced folate carrier;1 RFC1) which is a high-capacity, bi-directional transporter of reduced folate and also mediates the influx of TMP and efflux of TPP from cells (Zhao *et al.*, 2001). In addition, it actively transports folate analogues/derivatives such as the chemotherapeutic agent methotrexate.

The rs1051266 variant is located in exon 2 of *SLC19A1* (80G>A) and results in a histidine to arginine substitution at position 27 in the protein sequence. The estimated genotype frequencies of this SNP among Caucasians are: GG = 0.29, GA = 0.473, AA = 0.24 with an A allele frequency 0.47 (Rady *et al.*, 2001; Yee *et al.*, 2010). The function of this variant on transport uptake has been widely studied as has its association with drug responses and toxicity, particularly methotrexate, a folate antagonist used to treat malignancies and inflammatory conditions (Yee *et al.*, 2010). Thus, people with rheumatoid arthritis who are homozygous for the A allele show higher plasma concentrations of methotrexate and its active metabolites than their GA or GG counterparts (Dervieux *et al.*, 2004) and a higher probability of symptom remission (Drozdzik *et al.*, 2007). It also follows that homozygous carriers of the A allele are significantly more likely to experience methotrexate related toxicity.

An association was observed between TPP efflux in the presence of the *SLC19A1*:rs1051266:G variant and WKS in the present study. In addition, *in vitro* assays of the culture medium of cell lines overexpressing rs1051266:G, under conditions of low thiamine availability, contained significantly lower TPP concentrations than the culture medium of cells overexpressing rs1051266:A. This would

suggest that carriers of rs1051266:G might export less TPP than carriers of the rs1051266:A under conditions at least when thiamine supplies are low.

Human single-cell RNAseq data from multiple cortical areas available on the Allen Brain Map (https://portal.brain-map.org/) indicates that *SLC19A1* is preferentially expressed in pericytes. Expression in these cells is supported by the analysis of data from five independent mouse mRNA expression studies (He *et al.*, 2016). Pericytes are located in the vasculature of the brain and are involved in numerous processes including the maintenance of the blood-brain barrier (Armulik *et al.*, 2010). Whether *SLC19A1* expression in these cells facilitates TPP transport through the blood-brain barrier remains unknown; however, reduced efflux of TPP from these cells under low thiamine conditions could reduce the availability of TPP in the central nervous system.

Thiamine deficiency is common in people with alcohol dependence, but clinically apparent WKS is only observed in approximately 13% of these individuals, although the overall prevalence is acknowledged to be higher. *SLC19A1*:rs1051266:G has an estimated frequency of 0.50 in Caucasian populations. Thus, it is unlikely that it is the sole genetic factor influencing the risk for WKS. The possibility that *SLC19A1*:rs1051266: interacts with other genes variants involved in the thiamine metabolic pathway to increase the susceptibility to WKS needs to be considered. Interactions between common variants in the genes controlling the folate pathway have been shown to determine responses to methotrexate and sulfasalazine combination regimens in people with early rheumatoid arthritis (James *et al.*, 2008). Specifically, a gene-gene interaction between *SLC19A1* and *MTR* (5-methyltetrahydrofolate-homocysteine methyltransferase) has been documented; enhanced treatment responses are associated with joint carriage of *SLC19A1*:rs1051266:A and *MTR*:rs1805087:A (James *et al.*, 2008).

This study has several strengths. First, the definition of the cases and controls was based robustly on well-accepted criteria; the inclusion of a control population with a history of alcohol dependence offset the possible confounding effects of a possible genetic susceptibility to alcohol dependence *per se.* Second, functional studies were undertaken to determine the effect of the gene variant identified as nominally associated with WKS on thiamine metabolism; and third, the *in vitro* experiments were

conducted under thiamine deficient conditions replicating the situation found in people who develop WKS.

The study also has several limitations. First, the number of cases and controls was relatively small which limits the conclusions that can be drawn about the role played by *SLC19A1*:rs1051266 in determining susceptibility to WKS. This is further compounded by the high frequency of the rs1051266:A and rs1051266:G alleles **in the general population and therefore the genetic results may represent a false-positive finding. Second, the MMSE may also be insensitive to the detection of alcohol-related brain damage and no further information was available on other potential causes of brain damage or thiamine treatment amongst the control subject**. Third, reliable measures of intracellular TPP could not be obtained which would have provided a more granular insight into the role of TPP at the biological level *in vitro*.

WKS is frequently underdiagnosed and often inadequately treated. Identifying the factors which confer susceptibility to develop this condition would allow people at risk to be identified before they suffer irreversible brain damage. This study has contributed to the unravelling of the genetic component of disease susceptibility. Further larger studies including genome-wide association studies are warranted.

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Data Availability Statement

All data generated in this study are available from the corresponding author upon reasonable request.

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Conflict of Interest Statement

None declared. The funder had no contribution in designing experiments and preparing the manuscript

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Legend for Figure 1

Figure 1. Thiamine transport and metabolism pathways. Thiamine enters the cell via two transporters, SLC19A2 and SLC19A3. Within the cell, it is converted into its active form thiamine diphosphate (TPP) which is required for several essential metabolic pathways. The SLC19A1 transporter is involved in the efflux of TPP and the influx of folate monoglutamate. The key genes involved in these metabolic processes are shown in bold.

TABLE 1. Panel of genes used for the exome sequencing analysis in 29 cases with alcohol-related Wernicke-Korsakoff Syndrome.

Gene	Name	Pathway
ADH1B	Alcohol dehydrogenase 1B	Alcohol metabolism
ALDH2	Aldehyde dehydrogenase 2	Alcohol metabolism
APOE	Apolipoprotein E	WKS related
BCKDK	Branched chain ketoacid dehydrogenase kinase	Thiamine pathway
BCKDHA	Branched chain ketoacid dehydrogenase E1, alpha polypeptide	Thiamine pathway
DHTKD1	Dehydrogenase E1 and transketolase domain containing 1	Thiamine pathway
DLAT	Dihydrolipoamide S-acetyltransferase	Citric acid cycle
DLD	Dihydrolipoamide dehydrogenase	Citric acid cycle
DLST	Dihydrolipoamide S-succinyltransferase	Citric acid cycle
GABRA6	Gamma-aminobutyric acid (GABA) A receptor, alpha 6 subunit	Alcohol dependence
GABRA4	Gamma-aminobutyric acid type A receptor, alpha4 subunit	Alcohol dependence
GABRQ	Gamma-aminobutyric acid (GABA) A receptor, theta subunit	Alcohol dependence
GABRR1	Gamma-aminobutyric acid (GABA) A receptor, rho 1 subunit	Alcohol dependence
GKN2	Gastrokine 2	Thiamine pathway
HACL1	2-Hydroxyacyl-CoA lyase 1	Thiamine pathway
MCCC1	Methylcrotonoyl-CoA carboxylase 1 (alpha)	Thiamine pathway
OGDH	Oxoglutarate (alpha-ketoglutarate) dehydrogenase (lipoamide)	Citric acid cycle
OGDHL	Oxoglutarate dehydrogenase-like	Citric acid cycle
PCCB	Propionyl-CoA carboxylase beta subunit	Citric acid cycle
PDHA2	Pyruvate dehydrogenase (Lipoamide) Alpha 2	Citric acid cycle
PDHX	Pyruvate dehydrogenase complex, component X	Citric acid cycle
SLC19A1	Solute carrier family 19 (folate transporter), member 1	Thiamine pathway
SLC19A3	Solute carrier family 19, member 3	Thiamine pathway
SLC25A19	Solute carrier family 25 member 19	Thiamine pathway
THTPA	Thiamine triphosphatase	Thiamine pathway
TKT	Transketolase	Thiamine pathway
TKTL2	Transketolase-like 2	Thiamine pathway

Abbreviations: WKS, Wernicke-Korsakoff Syndrome.

TABLE 2. Genotype counts and test of association for selected exome-identified variants in alcohol-dependent subjects with WKS and alcohol-dependent subjects without cognitive impairment/brain damage

Gene, SNP, Effect	A1	A2	Case: exome sequenced (n = 29)		Cases: candidate genotyped (n = 87)		Controls* (n = 197)		Significance** (p)	FDR (p)
			Genotypes	AF	Genotypes	AF	Genotypes	AF		
ADH1B, rs1229984, His48arg	T	C	0/0/29	0.0	0/0/96	0.0	0/1/196	0.003	NA	
BCKDHA, rs11549936, Pro39his	A	C	0/9/20	0.155	2/17/69	0.119	4/65/123	0.19	0.089	0.626
BCKDK, rs144747578, Ser91ile	T	C	0/2/27	0.034	0/0/94	0.000	0/1/191	0.003	NA	
BCKDK, rs761419965, Arg399trp	T	C	0/1/27	0.018	0/0/94	0.000	0/0/190	0.000	NA	
DHTKD1, rs147571909, Val58ala	C	T	0/2/27	0.034	0/1/95	0.005	0/1/185	0.003	0.405	0.774
DLST, rs142872233, Pro118leu	T	C	0/3/26	0.052	0/2/91	0.011	0/6/188	0.015	0.841	0.994
GABRA4, rs115206335, Arg466gln	T	C	0/1/28	0.017	0/0/95	0.000	0/0/194	0.000	NA	
GABRA4, rs2229940, Leu26met	T	G	3/12/14	0.31	20/35/40	0.395	30/87/76	0.381	0.436	0.774
GABRA6, rs76773579, Gln157arg	G	A	0/1/27	0.018	1/1/93	0.016	1/1/191	0.008	0.396	0.774
GABRR1, rs1186902, His27arg	C	T	8/4/15	0.37	26/9/57	0.332	55/34/103	0.375	0.338	0.774
GABRQ, rs3810651, Phe478ile	T	A	10/2/17	0.375	40/5/50	0.447	59/22/114	0.359	0.1605	0.749
GKN2, rs62133344, Asp65tyr	A	C	1/5/23	0.121	0/12/77	0.067	3/18/173	0.062	0.754	0.994
OGDHL, rs11101224, Thr428met	A	G	0/9/19	0.155	3/30/63	0.188	7/61/118	0.202	0.526	0.817
PCCB, rs144907014, Ile189thr	C	T	0/2/27	0.034	0/0/88	0.000	0/1/188	0.003	NA	
PDHA2, rs138794638, Gly289ala	C	G	1/1/27	0.052	0/0/95	0.000	2/0/192	0.01	0.992	0.994
PDHX , rs149337104, Glu166Gln	C	G	0/1/28	0.017	0/0/95	0.000	0/0/194	0.000	NA	
TKT, rs139694567, Arg246gln	T	C	0/1/28	0.017	0/0/95	0.000	0/2/191	0.005	NA	
TKT, rs150551962,*** Asp200glu	T	G	0/0/28	0.000	0/0/95	0.000	0/4/190	0.01	0.988	0.994
TKTL2, rs11735477, Gln590his	A	T	0/9/20	0.155	3/24/67	0.16	4/54/133	0.162	0.442	0.774
TKTL2, rs150570593, Gln371x	A	G	0/1/27	0.018	0/0/93	0.000	0/0/191	0.00	NA	
THTPA, rs760779240, Thr148ser	T	A	0/1/28	0.017	0/0/94	0.000	1/0/192	0.005	0.994	0.994
SLC25A19, rs7213318,*** Glu113asp	A	G	0/0/29	0.000	0/0/95	0.000	0/2/192	0.005	NA	
SLC19A1, rs1051266, His27arg	A	G	3/19/7	0.431	17/42/33	0.413	47/93/50	0.492	**0.01**	0.144

*Control Cohort of alcohol dependent individuals with no clinical features suggestive of WKS or other forms of brain injury scoring > 26 on the Mini Mental score 24; **logistic regression with sex as a covariate; *** The exome sequencing data for these variants was not confirmed in the genotyping assay; Abbreviations: WKS Wernicke-Korsakoff Syndrome; SNP: single nucleotide polymorphism; A1 Allele 1; A2 Allele 2; AF, A1 allele frequency; Significance of association emboldened values indicate $p < 0.05$; FDR p false discovery rate significance

TABLE 3. The impact of *SLC19A1* rs1051266 alleles on thiamine pyrophosphate levels in cell culture media after 24-hour incubation under different conditions

Media Conditions	WKS Risk Allele (G)		Alternate Allele (A)		Control Cells		Significance * (p)			
	Mean AUC	TPP (nM)	Mean AUC	TPP (nM)	Mean AUC	TPP (nM)	G vs A	G vs control	A vs control	G vs A all thiamine concentrations
0% FBS 0.00 nM Thiamine	0.87	15.65	2.11	37.77	0.59	10.66	$5.0x10^{-04}$	$7.2x10^{-02}$	$3.3x10^{-06}$	$5.7x10^{-11}$
0% FBS 0.005 nM Thiamine	0.78	13.92	1.48	26.55	1.20	21.49	$2.0x10^{-04}$	$3.4x10^{-03}$	$4.8x10^{-03}$	
0% FBS 0.05 nM Thiamine	0.80	14.34	1.71	30.65	1.23	22.04	$5.9x10^{-06}$	$2.7x10^{-03}$	$2.1x10^{-03}$	
0% FBS 0.5 nM Thiamine	0.84	15.08	1.72	30.75	1.91	34.21	$2.1x10^{-03}$	$1.6x10^{-03}$	$3.6x10^{-01}$	
0.2% FBS 0.00 nM Thiamine	1.25	22.48	2.69	48.17	1.80	32.34	$2.6x10^{-05}$	$7.2x10^{-04}$	$2.6x10^{-04}$	$2.9x10^{-04}$
0.2% FBS 0.005 nM Thiamine	1.29	23.19	2.58	46.22	1.87	33.46	$1.5x10^{-05}$	$7.6x10^{-03}$	$3.5x10^{-03}$	
0.2% FBS 0.05 nM Thiamine	1.34	23.98	1.55	27.78	1.18	21.16	$2.5x10^{-02}$	$7.2x10^{-02}$	$5.8x10^{-04}$	
0.2% FBS 0.5 nM Thiamine	1.77	31.66	1.71	30.72	1.46	26.14	$6.2x10^{-01}$	$3.2x10^{-02}$	$1.2x10^{-01}$	

Data are presented as mean thiamine pyrophosphate concentration from three independent experiments.
*Comparison of the areas under the curve (AUC) for G and A alleles under all media conditions.
Abbreviations: WKS: Wernicke-Korsakoff Syndrome; AUC: area under concentration–time curve; TPP: thiamine pyrophosphate

TABLE S1. Effect of thiamine deficiency at three time points on the viability of the HEK293 cells compared to normal cell culture conditions. Fetal Bovine Serum contains thiamine and is included as a variable factor in this experiment in addition to thiamine.

Time point	FBS in media (%)	Final concentration of exogenous thiamine (nM)								Mean *	Viability of Cells** (%)
		0	0.005	0.01	0.05	0.1	0.5	1	5		
24 hr	0	374	321	450	336	316	61	307	250	302	-48
	0.2	486	628	600	774	637	251	553	413	543	-6
	0.4	564	743	865	667	879	424	742	464	669	16
	0.6	546	740	755	748	822	486	525	411	629	9
	0.8	629	632	834	618	681	404	776	314	611	6
	1	591	749	611	754	819	557	773	488	668	16
	2	733	758	765	680	626	500	683	664	676	17
	2.2	638	527	673	606	698	397	479	597	577	0
48 hr	0	115	173	176	154	235	169	168	326	190	-88
	0.2	353	517	673	684	462	702	744	61	554	-65
	0.4	382	492	832	920	761	890	967	531	722	-54
	0.6	565	593	1018	829	919	849	963	625	795	-49
	0.8	697	621	965	947	835	816	990	714	823	-48
	1	738	855	880	1038	930	943	1116	846	918	-42
	2	1281	1464	1749	1678	1874	1602	1703	1099	1556	-1
	2.2	1165	1526	1681	1651	1704	1756	1657	1449	1574	0
72 hr	0	340	252	271	203	213	245	222	232	247	-88
	0.2	1001	845	834	785	1021	836	791	602	839	-58
	0.4	826	1199	870	980	758	857	959	735	898	-55
	0.6	1016	986	1216	782	971	623	718	648	870	-56
	0.8	898	1072	761	816	661	711	926	1115	870	-56
	1	918	1305	1231	975	1014	870	1027	1385	1091	-45
	2	2250	3118	2609	2348	2781	2535	2701	1832	2522	27
	2.2	1729	2112	1942	1903	2120	2303	2310	1413	1979	0

Data are presented as the mean cell counts from three individual experiments.

* Mean of all thiamine concentrations

** Comparison with reference viability in 2.2% FBS

Abbreviation: FBS: foetal bovine serum

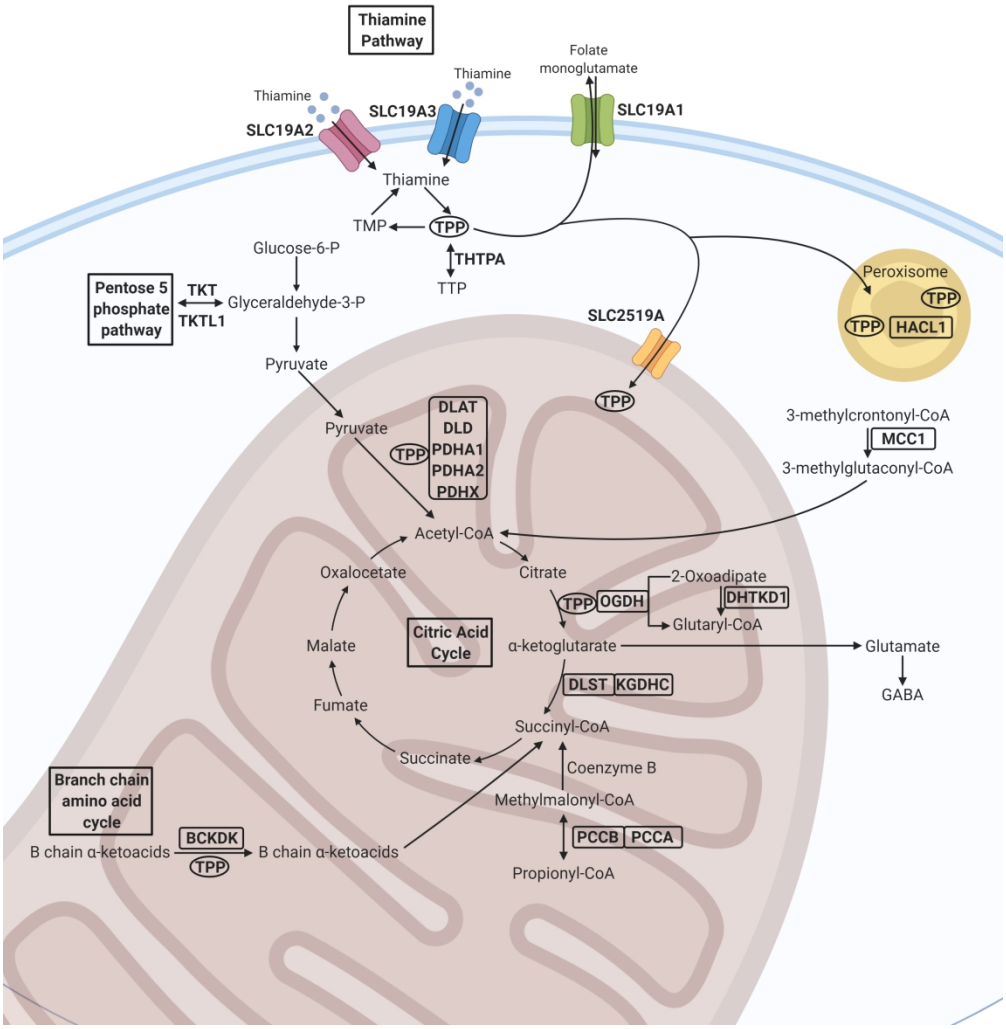

Figure 1. Thiamine transport and metabolism pathways. Thiamine enters the cell via two transporters, SLC19A2 and SLC19A3. Within the cell, it is converted into its active form thiamine diphosphate (TPP) which is required for several essential metabolic pathways. The SLC19A1 transporter is involved in the efflux of TPP and the influx of folate monoglutamate. The key genes involved in these metabolic processes are shown in bold.

304x355mm (300 x 300 DPI)