

Risk Association of MTHFR Polymorphisms for Severity of the Disease in Children with Sickle Cell Disorder

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Abstract

The MTHFR variants, C677T and A1298C have been reported to contribute towards higher severity in sickle cell disorder. Hence the study was conducted on children with sickle cell disease to find out the association of the genotypes of MTHFR and disease severity. A cross-sectional analytical study was conducted on 249 children aged 5–15 years, diagnosed with sickle cell disorder. Severity Index was assigned to each child based on the clinical history of frequency of episodes of painful crisis, vascular crisis, joint pain, hemoglobin levels and hospitalization required. The children were grouped as $SI \leq 6$ (Mild) and $SI > 6$ (Severe). The study participants comprised of 221 (88.76%) and 28 (11.24%) children in $SI \leq 6$ and $SI > 6$ respectively. The SI score in the children was significantly associated with the MTHFR genotype variants C677T ($p = 0.000$), A1298C ($p = 0.049$), plasma homocysteine levels ($p = 0.03$), blood hemoglobin values ($p = 0.000$) and frequency of hospitalization ($p = 0.000$). Weight ($p = 0.006$), BMI ($p = 0.000$) and hemoglobin levels ($p = 0.000$) were significantly lower in children with $SI > 6$ whereas plasma homocysteine was found higher ($p = 0.000$). Genotypes CT and TT showed higher odds of $SI > 6$ as compared to CC genotype and genotype C'C' showed higher odds of having $SI > 6$ as compared to genotypes AA and AC' combined. The genotypes CC-AC', CT-C'C' and TT-AA were found to be associated with $SI > 6$. The study reported significant association of MTHFR polymorphisms, C677T and A1298C with severity index score in children with sickle cell disorder. The variants forms of the studied SNPs (TT and C'C') were found to have significant implication with the severity of the disease.

Introduction

5,10-methylene tetrahydrofolate reductase (MTHFR) is a key enzyme involved in folate metabolism that catalyses the conversion of 5,10 methylenetetrahydrofolate to 5-methyltetrahydrofolate, the principal circulatory form of folate. It plays key role in methionine-homocysteine cycle in the cells and production of S-adenosyl methionine, an essential methyl donor for biosynthesis of nucleic acid, proteins, neurotransmitters and many other bioactive molecules required for cell division and cell growth. Reduced activity of MTHFR is proposed to be a genetic risk factor for hyperhomocysteinemia, reduction in methylation of biological molecules and increase in non-methylated folate [1].

Among various polymorphic forms, two most common single nucleotide polymorphisms (SNPs), C677T and A1298C have been reported to contribute towards the occurrence of thromboembolic phenomenon in sickle cell disorder [2–4]. Studies in various regions of India reported prevalence rate of C677T polymorphism to be 10% – 20% and that of A1298C to be 20% – 30% [4–8]. Study at the Regional Medical Centre (ICMR) in Jabalpur, Madhya Pradesh depicted significantly higher prevalence of mutant variants of MTHFR gene (28% heterozygotes and 14.6% homozygotes) in sickle cell patients as compared to normal /control individuals [9]. Recent studies have proposed that presence of these mutant variants showed higher incidence of pain in chest, abdomen, and bone joints along with early age of onset of clinical manifestations as well as frequent dependence on blood transfusion in these group of patients. Individuals carrying mutated MTHFR gene had shown significant association with higher levels of homocysteine which has been regarded as an important risk factor for stroke and other

thromboembolic phenomenon. Thus, investigators have suggested that these variants might be a risk factor for occurrence of vascular events and higher severity in sickle cell cases [4, 10, 11]. However, the combined effect of both the MTHFR polymorphisms has not been widely studied in children with sickle cell disorder.

The prevalence of sickle cell disorder in Chhattisgarh state is around 10%. Because of limited data available for MTHFR genotypes and high prevalence rate of sickle cell anemia in this state, this study would be the first of its kind to furnish substantial information regarding the association of the genotypes of MTHFR and disease severity in this region of Central India.

Materials And Methods

A cross-sectional analytical study was conducted on 249 children of age group 5–15 years, diagnosed with sickle cell disorder attending our institute. Both, heterozygous (AS) sickle cell trait (SCT) and homozygous (SS) sickle cell disease were recruited for the study. The study was approved by the Institute Ethics Committee and written informed consent from the parents/legal representatives before enrollment. Demographic details and clinical details were entered in pre-structured questionnaire. The details included family history, previous clinical history and frequency of clinical severity or hospitalizations required due to any acute crisis in past one year. Each child was assessed for clinical severity based on the clinical history of frequency of episodes of painful crisis, vascular crisis, joint pain, hemoglobin levels and hospitalization required in that one year period.

Severity Index (SI) score, was assigned to each children based on the clinical history of frequency of episodes of painful crisis, vascular crisis, joint pain, hemoglobin levels and hospitalization required. The children were grouped as $SI \leq 6$ (Mild) and $SI > 6$ (Severe) [12–14].

Under all aseptic precautions, 3 ml of blood was collected in ethylenediaminetetraacetic acid (EDTA) vacutainers. Half of the collected blood was aliquoted and centrifuged to separate the plasma for homocysteine analysis by chemiluminescence method in Advia Centaur XP Fully Automated Chemiluminescence Immunoanalyzer, Siemens. 1 ml of the remaining blood was processed for hemoglobin estimation in XP-100 Fully Automated hematology Analyzer, Sysmex.

The remaining 500µl blood was processed for deoxyribonucleic acid (DNA) extraction using Invitrogen™ PureLink™ Genomic DNA Mini kit from ThermoFisher Scientific. The two SNP Genotypes, C677T (rs1801133) and A1298C (rs1801131) (Assay ID's C_1202883_20 and C_850486_20 respectively) were processed with pre-validated TaqMan SNP Genotyping Kit polymerase chain reaction (PCR) assays from Applied Biosystems, ThermoFisher (Foster City, CA) on CFX96 real time PCR system, Biorad (USA). The prototype images for TaqMan Real time PCR are attached below as fig_{suppl}.1 to 4 and that of allelic discrimination plot of the genotypes as fig_{suppl}.5.

The genotypes for C677 were categorized as homozygous wild (CC), heterozygous (CT) and homozygous variant (TT). T allele was considered as the Mutant allele for the said SNP.

Similarly, the genotype for A1298 were homozygous wild (AA), heterozygous (AC') and homozygous variant (C'C'). C' allele was considered as the Mutant allele for this genotype. C' has been used instead of C for A1298C genotype so that it can differentiate the said allele from C allele of C677T. The same would be noted in the manuscript henceforth.

The statistical analysis was carried out in SPSS version 20. The association of the two groups with the demographic, clinical and genotype variables was analyzed using Chi-square test or Fisher's exact test. Independent t-test was used for comparison of the quantitative variables between the groups. Odds Ratio (OR) for combined genotypes contributing towards SI was calculated using 2x2 table using Chi-square test. For all statistical calculations, p-value less than 0.05 was considered significant.

Results

The mean (\pm SD) age group of the study population is 11.27(\pm 3.47) years. Total 249 children were enrolled for the study out of which 218 (87.6%) were AS (SCT) and 31(12.4%) were SS (SCD). The study participants were grouped under $SI \leq 6$ and $SI > 6$, comprising of 221 (88.76%) and 28 (11.24%) children respectively.

The graphical representation of association of the study groups with the variables is shown in figure-1. A significant association of severity index was observed with all the variables except for age group, gender and height. The SI was found to be associated with the sickle cell status of the patient, AS & SS, ($\chi^2 = 11.11$, $p = 0.001$); weight percentile ($\chi^2 = 9.9$, $p = 0.007$) and BMI percentile ($\chi^2 = 12.996$, $p = 0.002$) of the children. The SI score in the children under study was found to be dependent on the MTHFR genotype variants C677T ($\chi^2 = 20.599$, $p = 0.000$) and A1298C ($\chi^2 = 6.039$, $p = 0.049$). Plasma homocysteine levels ($\chi^2 = 4.725$, $p = 0.03$), blood hemoglobin values ($\chi^2 = 16.645$, $p = 0.000$) and frequency of hospitalization ($\chi^2 = 129.93$, $p = 0.000$) significantly associated with SI.

The weight, BMI, Hb and homocysteine levels differed significantly between the groups (table-2). Weight ($p = 0.006$), BMI ($p = 0.000$) and hemoglobin levels ($p = 0.000$) were significantly lower in children with $SI > 6$ whereas plasma homocysteine was found higher ($p = 0.000$) in this group.

The distribution and association of the MTHFR variants in the groups is depicted in table-2. The frequency of CC, CT and TT genotypes of C677T was 71.9%, 26.1% and 2.0% respectively in the study population. The frequencies for A1298C SNPs were 30.9%, 49.4% and 19.7% for AA, AC' and C'C' respectively. The frequency distribution within the study population did not deviate Hardy-Weinberg Equilibrium (HWE) for C677T ($\chi^2 = 0.999$, $p = 0.95$) and A1298C ($\chi^2 = 0.39$, $p = 0.44$). The genotype C677T was significantly associated with $SI > 6$. Genotypes CT and TT showed higher odds of $SI > 6$ in sickle cell cases as compared to CC genotype. The odds of $SI > 6$ for CT genotype was 2.817 times (1.259–6.301) as compared to the other two genotypes. CC genotypes seems to have a protective odds of 76 % (45% -90%) against a $SI > 6$. Having mutant allele T seemed to be significantly associated with a $SI > 6$ and a 3.533 times (1.905–6.578) increased odds of having $SI > 6$ in the study population as compared to having the wild C allele of C677T SNP. Genotype A1298 also reflected significant association with SI

score in this study. The genotype C'C' had 2.593 times higher odds of having SI > 6 as compared to genotypes AA and AC' combined. The alleles A and C' of A1298C did not show any association with SI in the study subjects.

The risk association of the combined genotypes for severity index in sickle cell children is depicted in table-3. The genotypes CC-AC', CT-C'C' and TT-AA were found to be associated with SI > 6. The increased odds of having a SI > 6 was 6.199 times (1.733–23.27) for CT-C'C' genotype. Similarly, the odds for TT-AA was 18.89 times (1.062-undefined) as compared to CCAA but the smaller sample count for the said genotype limits the approximate prediction of an odds ratio.

Discussion

The cross-sectional study was conducted on 249 children diagnosed with sickle cell disorder. The mean (\pm SD) age of the study subjects attending the Sickle Cell Unit of our institute was 11.27 (\pm 3.47) years. 11.24% of the study population scored for SI > 6 during enrollment. Nearly two times frequency of 63.7% ($n = 65$) severe (SI > 6) and 36.3% ($n = 37$) mild cases were observed in 102 children with homozygous (SS) SCD cases in Abdul-Wahab M et al study. The frequency of severity in females ($p < 0.01$) and after 5 years ($p < 0.01$) was more [14]. Our results contradicted as no age and gender discrimination was observed in the study population (fig-1).

The HWE of the polymorphisms was not violated. Very limited studies are available for both MTHFR polymorphisms in sickle cell disorder. This is one of its kinds to show the genotypic interaction of these polymorphisms in children with sickle cell disorder in this region. The genotype frequencies were comparable to Butler et al study that reported 34.6% for CT, 3% for TT, 35.9% for AC' and 20.8% for C'C' in North Indian population with coronary artery disease (CAD) [8]. The prevalence of polymorphisms in Nigerian population was 19.3% (C677T) and 15% (A1298C) whereas in Tunisian population it was 76.56% and 15.6% respectively [15, 16]. Nishank et al study presented 28% and 14.6% prevalence for CT and TT in Central India (Madhya Pradesh) [9]. The polymorphisms are widely distributed and show varied frequencies as per geographical location and ethnicity of the study population depending on the genetic and environmental interaction [17]. The severity score of the enrolled children depicted association with MTHFR genotypes. CT and TT variants of C677T and C'C' variant of A1298C SNP showed significant implication on SI. This shows that C' allele in homozygous state has an impact on the severity as compared to heterozygous genotype. Hatzlhofer et al study on 277 SCA in Pernambuco, Brazil cases reported significant risk of C677T variants ($p = 0.015$) for vascular complications but no association for MTHFR A1298C SNP [3]. Lakkakula et al meta-analysis showed increased risk for vascular crisis in sickle cell disorder with MTHFR C677T (CT + TT vs CC, $p < 0.001$) [10]. Al-Absi et al study differed by reporting significant association of A1298C genotype with sickle cell disease ($p = 0.03$) but not C677T SNP in 106 Bahraini patients [18]. Kangne et al study also found no association of C677T polymorphism with vascular phenomenon [19]. In our study, the CT-C'C' children registered nearly 6.19 times and those with TT-AA depicted 18.89 times risk for high severity index than the wild CC-AA children. The differences in the level of enzyme activity reduction could be due to the varied attenuation effects of the

polymorphisms. Few investigators postulated the decrease in enzyme activity by 60% and 35% respectively for C677T and A1298C SNPs [8, 20]. The cellular methionine-homocysteine cycle is influenced not by the cellular availability of folates and B12 but also by the activity of the enzyme MTHFR. Reduced folate or B12 levels or MTHFR activity would lead to homocysteinemia which in turn related to pathogenesis and clinical manifestations in sickle cell disorder [2, 4, 8]. The present study accorded significant implication of plasma hyperhomocysteinemia on the clinical severity of the disease. The study by Al-Absi et al reported no association of disease condition with raised plasma homocysteine levels and suggested for larger sample size for confirming the role of homosysteine in sickle cell disease [18]. The difference in sample size might be accounted for the contradictory findings as our sample size was more than double to that of Al-Absi et al study. Though hyperhomocysteinemia have been ascribed to low plasma folate and B12 levels but homocysteine levels were increased in 71.5% of our study population even if all the children were under folic acid supplementation. This could be the impact of the significant association of C'C' and TT with the severity of the disorder. Risk for higher SI was more in presence of both mutant alleles in an individual as shown in table-2. The homozygous genotype for mutant allele, AA-TT and C'C'-CT genotype indicated 18.89 times and 6.199 times risk for SI > 6. Wahab-Abdul et al study observed no association between TT genotype and homocysteinemia or severity of disease in 102 SS children. This was justified by the fact that the enrolled children were supplemented with folic acid, B6 and B12 and no differences in the plasma values of these vitamins were reported. Hence, it was proposed that supplementation of folic acid with B12 and B6 might overcome the effect of mutant allele [14]. The shortcoming of the present study was that plasma B12 levels were not measured in the children to rule out the effect of these genotypes on homocysteine levels. Moreover, the homocysteine level was measured in plasma at the time of attending the OPD with clinical manifestations. Our institute being a tertiary care center usually caters patient influx with some form of severity or when treatment measures fail at other centers. This also explains the fact that children with SI > 6 had significantly lower hemoglobin values at the time of presentation (table-1). Altered folate-B12 metabolism, anemia and presence of genetic factors in the children might also affect the optimal growth of the children leading to low BMI (82.1%) and higher frequency of hospitalization (96.4%) in severe group as revealed in our study.

Conclusion

Our study reported significant association of MTHFR polymorphisms, C677T and A1298C with severity index score in children with sickle cell disorder. The variants forms of the studied SNPs (TT and C'C') were found to have significant implication with the severity of the disease. Hence, screening for these variants might aid in identifying the risk groups for severe disease conditions. However, limitations reside in the fact that we did not measure the plasma levels of folic acid and B12 in these children to rule out their effect on plasma homocysteine levels and the pathophysiological relevance. Further investigations including control group and children with sickle cell disorder without any clinical manifestation might provide an accurate association of these genotypes to severity of the disease.

Declarations

Funding info

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Conflicts of Interest : No potential conflict of interest relevant to this article was reported.

Ethics approval

The study was initiated after approval from Institute Ethics Committee (IEC).

Consent to Participate:

All participants were enrolled after the parents/legally accepted representatives signed the informed consent approved by IEC.

Consent to Publish

The study was approved by IEC for publication provided participant's confidentiality is maintained.

Author's contribution

Concept and design – Dr S. Patel, Dr. S. Kumar; Data acquisition and lab procedures – Dr S. Patel, Dr. N Hussain, Prof. P. K. Patra; Data analysis and interpretation – Dr. G. Naik, Dr R. Nanda; Drafting manuscript – Dr S Patel, Dr S. Kumar; Revising manuscript for critically important intellectual content – Prof. E. Mohapatra, Prof. P.K. Patra

All authors approved the final version of the manuscript to be published.

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Tables

Table-1: Comparison of mean values of the variables in the study groups

Variables	SI ≤ 6	SI > 6	t-value	p-value	95%CI	
					Lower	Upper
Age(years)	11.37 ± 3.46	10.42 ± 3.53	1.303	0.19	– .464	2.277
Weight (Kg)	31.38 ± 11.73	28 ± 8.48	2.772	0.006	1.838	10.860
Height (cm)	136.62 ± 16.81	131 ± 16.39	1.670	0.096	-1.007	12.238
BMI	16.12 ± 2.56	14.13 ± 1.64	4.003	0.000	1.011	2.969
Hb levels (g/dL)	10.37 ± 1.33	8 ± 1.30	8.921	0.000	1.848	2.895
Homocysteine levels (μmol/L)	19.81 ± 9.67	33.5 ± 16.27	6.443	0.000	-17.881	-9.508

Table-2: Risk association of MTHFR variants towards severity of the disease

Genotype	SI ≤ 6 N(%)	SI > 6 N(%)	Comparison between the genotypes for SI > 6	OR (95% CI)	Chi square test (p- value)
C677T					
CC	167(75.6)	12(42.9)	CC vs CT + TT	0.243 (0.108– 0.545)	13.156 (0.000)
CT	52(23.5)	13(46.4)	CT vs CC + TT	2.817 (1.259– 6.301)	6.756 (0.009)
TT	2(0.9)	3(10.7)	TT vs CC + CT	13.140 (2.094– 82.440)	12.153 (0.011) (Fischer Exact)
Fischer Exact test (p- value)	15.28 (0.000)				
C	386(87.3)	37(66.1)	T vs C	3.533 (1.905– 6.578)	17.56 (0.000)
T	56(12.7)	19(33.9)			
Chi square test (p- value)	17.56 (0.000)				
A1298C					
AA	68(30.8)	9(32.1)	AA vs AC'+C'C'	1.066 (0.459– 2.476)	0.022 (0.882)
AC'	114(51.6)	9(32.1)	AC' vs AA + C'C'	0.445 (0.193– 1.026)	3.758 (0.053)
C'C'	39(17.6)	10(35.7)	C'C' vs AA + AC'	2.593 (1.112– 6.047)	5.132 (0.023)
Chi square test (p- value)	6.039 (0.049)				
A	250(56.6)	27(48.2)	C' vs A	1.398 (0.801– 2.439)	1.403 (0.236)
C'	192(43.4)	29(51.8)			
Chi square test (p- value)	1.403 (0.236)				

Table-3: Risk association of the combined genotypes of MTHFR C677T and A1298C in the study populations towards severity index in children of sickle cell disorder

Combined Genotypes	SI > 6 N (%)	SI ≤ 6 N (%)	OR (95%CI)	Chi square test/ Fisher's exact (p- value)
CC-AA	45(10.2)	7(12.5)	Reference	-
CC-AC'	91(20.6)	3(5.4)	0.214 (0.043–0.856)	5.5 (0.009)
CC-C'C'	31(7)	2(3.6)	0.418 (0.056–2.025)	1.168 (0.14)
CT-AA	23(5.2)	0(0)	0.315(0.013–2.254)	2.324 (0.063)
CT-AC'	21(4.8)	5(8.9)	1.5 (0.398–5.509)	0.44 (0.253)
CT-C'C'	8(1.8)	8(14.3)	6.199(1.733–23.27)	9.5 (0.001)
TT-AA	0(0)	2(3.6)	18.89 (1.062-undefined)	7.66 (0.002)
TT-AC'	2(0.5)	1(1.8)	3.12 (0.09–45.86)	0.901 (0.171)
TT-C'C'	0(0)	0(0)	undefined	1.078 (0.150)

Figures

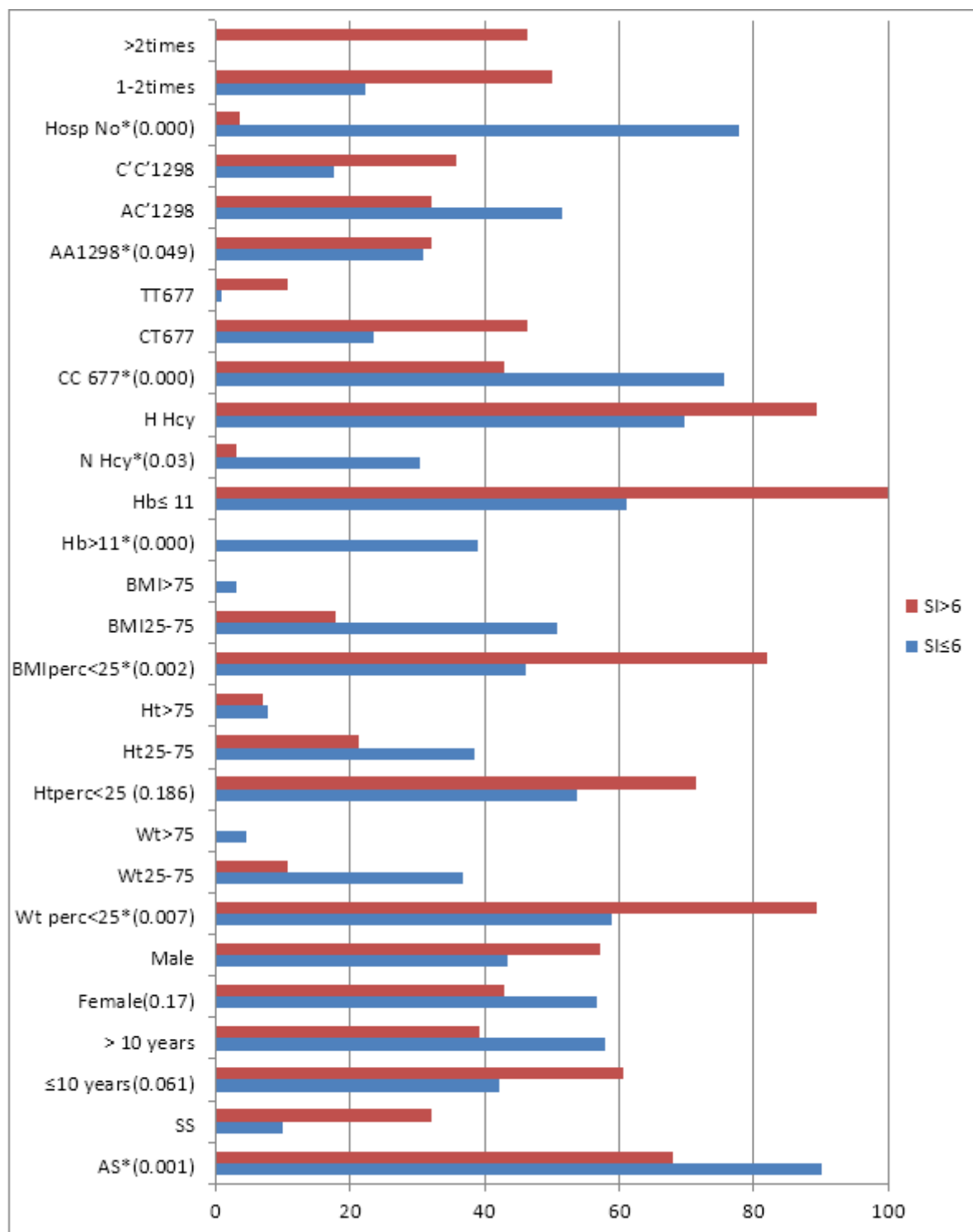


Figure 1

Graph showing association of the study groups with the categorical variables

Supplementary Files

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