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1 **Biallelic mutations in *P4HTM* cause syndromic obesity**

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36 **ABSTRACT**

37 We previously demonstrated that 50% of children with obesity from consanguineous families  
38 from Pakistan carried pathogenic variants in known monogenic obesity genes. Here, we have  
39 discovered a novel monogenetic recessive form of severe childhood obesity, using an in-  
40 house computational staged approach. This included analysis of whole-exome sequencing  
41 data of 366 children with severe obesity, 1,000 individuals of the Pakistani PROMIS study,  
42 and 200K participants of the UK Biobank, to prioritize genes harbouring rare homozygous  
43 variants with putative effect on human obesity. We identified five rare or novel homozygous  
44 missense mutations predicted deleterious in five consanguineous families in *P4HTM* encoding  
45 Prolyl 4-Hydroxylase Transmembrane (P4H-TM). We further found two additional  
46 homozygous missense mutations in children with severe obesity of Indian and Moroccan  
47 origin. Molecular dynamics simulation suggested that these mutations destabilized the active  
48 conformation of the substrate binding domain. Most carriers also presented with hypotonia,  
49 cognitive impairment and/or developmental delay. Three of the five probands died of  
50 pneumonia during the ~2 years of the follow up. *P4HTM* deficiency is a novel form of  
51 syndromic obesity affecting 1.5% of our children with obesity associated with high mortality.  
52 P4H-TM is a hypoxia inducible factor that is necessary for survival and adaptation under  
53 oxygen deprivation but the role of this pathway in energy homeostasis and obesity  
54 pathophysiology remains to be elucidated.

## 55 INTRODUCTION

56 Previously, our genetic studies on children with severe obesity from consanguineous families  
57 of Pakistan (severely obese Pakistani population/SOPP cohort), enabled a successful genetic  
58 diagnosis in ~50% of obese participants (1-5). This predominantly includes identification of  
59 pathogenic mutations in genes that play a key role in the hypothalamic leptin-melanocortin  
60 pathway (e.g. *LEP*, *LEPR* and *MC4R*), regulating appetite and energy homeostasis. Early-  
61 onset, severe obesity accompanied with severe hyperphagia are the most common phenotypes  
62 caused by these mutations.

63 There are also several well-known pleiotropic monogenic obesity syndromes where obesity is  
64 one of a number of clinical anomalies often including intellectual disability, structural  
65 abnormalities, dysmorphic features, vision and hearing impairments, maladaptive behaviour,  
66 and organ- and cell-specific anomalies, as observed in Bardet-Biedl, Alstrom, Carpenter and  
67 Cohen syndromes (6), amongst others. Our endeavour of unravelling genetic causality in  
68 SOPP also led to the identification of probands with these obesity syndromes (7) and also  
69 uncovered that bi-allelic mutations in *ADCY3* are a novel genetic cause of syndromic obesity  
70 (8).

71 Aiming at a further investigation of the genetic basis of severe obesity and to identify  
72 additional causes of obesity in undiagnosed cases from SOPP, we developed and employed an  
73 in-house systematic analysis of the sequencing data using MiST method (9) in combination  
74 with rigorous and step-wise data filtration. Additionally, we carried out molecular dynamics  
75 (MD) simulations to determine consequences of the mutations on the encoded proteins'  
76 stability and conformation. As a result, we have identified several patients with severe obesity  
77 carrying homozygous, deleterious, missense mutations in *P4HTM* encoding the enzyme prolyl  
78 4-hydroxylase transmembrane (P4H-TM), an atypical member of the hypoxia inducible  
79 factors prolyl 4-hydroxylases (HIF-P4Hs), that are necessary for adaptation under reduced

80 oxygen supply. This is the only member of HIF-P4Hs that predominantly expresses in brain  
81 but mechanism by which P4H-TM causes obesity is still unknown, opening new avenues of  
82 research in the physiology of energy balance.

83

## 84 **MATERIALS AND METHODS**

### 85 *Genetic and Statistical analysis*

86 Initially, all probands with severe obesity from SOPP ( $n=456$ ) were systematically screened  
87 for mutations in *LEP* and *MC4R* through Sanger sequencing. The probands found negative for  
88 pathogenic or likely pathogenic mutations in these two genes, were further analysed either  
89 through conventional whole-exome sequencing or augmented whole exome sequencing  
90 (CoDE-seq) described in detail elsewhere (10; 11).

91 Analysis of exome data was carried out stage-wise: **Stage I** All the genes known to be linked  
92 with monogenic obesity including syndromic obesity, were searched for pathogenic or likely  
93 pathogenic mutations by following the rules and guidelines of the American College of  
94 Medical Genetics and Genomics (**Supplementary Table 1**) (12). **Stage II** Gene-centric  
95 analysis using the MiST method was performed (9) on exome data from severely obese  
96 subjects with yet undiagnosed genetic causality, in SOPP ( $n=366$ ) and 1,003 normal-weight  
97 subjects from PROMIS (Pakistan Risk of Myocardial Infarction Study) cohort. Participants in  
98 both groups belong to the same geographical region allowing for a better comparison, to  
99 identify genes harbouring a significant burden of rare (*i.e.* with a minor allele frequency  
100 below 1%), homozygous and potentially deleterious (according to SIFT and PolyPhen)  
101 variants among the cases with obesity. In **Stage III**, the genes identified with a significant p-  
102 value at **Stage II** (*i.e.*  $P_{\pi} < 0.05$ ) were then investigated against 200K exome data from UK  
103 Biobank (Application #67575), where we assessed the association between null variants (*i.e.*

104 nonsense, frameshift, canonical  $\pm 1$  or 2 splice sites, initiation codon) per gene and body mass  
105 index (BMI) using MiST. Our statistical analyses were adjusted for age, gender and the first 5  
106 genetic principal components, to take into account any potential ethnicity confounds in the  
107 cohort. The details of the MiST analysis have been provided elsewhere (13). In the **Stage IV**,  
108 only the genes with mutations in 3 or more unrelated families were considered as to be likely  
109 involved in human obesity. Finally, in **Stage V**, literature survey and further clinical  
110 investigation of the affected probands was carried out (**Figure 1**).

### 111 **Biochemical analysis**

112 Metabolic markers including leptin, insulin and cortisol were determined using commercially  
113 available ELISA kits (Monobind, Lake Forest).

### 114 **Molecular modelling**

115 The molecular modelling was carried out for four of the seven mutations (p.Glu155Lys,  
116 p.Val297Met, p.Val316Ile and p.Gly433Ala), where crystal structural information was  
117 available. Here the dynamic and structural consequences of the P4HTM mutations on  
118 substrate binding were assessed through homology modelling followed by molecular  
119 dynamics (MD) simulations. The detailed procedure is given in **Supplementary**  
120 **Information**.

### 121 ***P4HTM* expression in murine and human hypothalamus**

122 Recently we generated an integrated single-cell atlas, namely HypoMap, of the murine  
123 hypothalamus. It consists of 384,924 cells collected from 18 different single-cell studies,  
124 covering various regions of the hypothalamus(14). The publicly available Seurat data object  
125 was used to assess the expression of *P4htm* in cells captured within the atlas.

126 To examine the expression for *P4HTM* in human hypothalamus, we extracted the  
127 hypothalamic cells from a single-nucleus sequencing dataset from adult human brains (15),  
128 available via the Neuroscience Multi-omics Archive (NeMO, RRID:SCR\_016152). Briefly,  
129 the count data from 134,471 nuclei labelled ‘hypothalamus’ from ROIGroupCoarse was  
130 extracted from the original loom file, and the count table was then imported into Seurat 4.1.1  
131 for log-normalisation; variable feature selection (3000 genes); data reduction via principal  
132 component analysis (PCA, 90 PCs); and uniform manifold approximation projection (UMAP  
133 using PCs 1:50) was used for data visualisation.

134

135 **RESULTS**

136 The stepwise analysis and rigorous filtration criteria (**Figure 1**) resulted in the identification  
137 of only one gene putatively involved in monogenic obesity: *P4HTM* (encoding Prolyl 4-  
138 Hydroxylase, Transmembrane). Through gene-centric analysis the burden of rare,  
139 homozygous, and potentially deleterious variants in *P4HTM* was significantly associated with  
140 obesity risk ( $P_{\pi} = 0.013$ ;  $\pi_{\text{hat}} = 14$ ; 95% CI<sub>2.5</sub> = -26;  $P_{\tau} = 1.0$ ). In the next step (*i.e.* Stage  
141 III) using the 200K exome data from UK Biobank, we assessed the association between null  
142 variants (**Supplementary Table 2**) in *P4HTM* and adiposity. We found that these null  
143 variants were significantly associated with higher BMI levels ( $P_{\pi} = 0.017$ ;  $\pi_{\text{hat}} = 1.4$ ; 95%  
144 CI 0.25–2.5;  $P_{\tau} = 0.89$ ). Notably, all the null variants that were detected in the UK biobank  
145 were heterozygous.

146 A total of seven mutations were identified in *P4HTM* in seven unrelated probands (all males)  
147 in consanguineous families. From the SOPP cohort, five different homozygous missense  
148 mutations (p.Pro45Leu, p.Val297Met, p.Val316Ile, p.Gly433Ala and p.Arg509Pro) were  
149 identified in 5 unrelated probands (**Table 1; Supplementary Figure 1**). A further screening  
150 of *P4HTM* in other cohorts identified 2 additional subjects with severe obesity carrying  
151 homozygous missense mutations (p.Gly116Asp and p.Glu155Lys), from consanguineous  
152 families of Indian and Moroccan origin, respectively. All the mutations identified and  
153 reported here are either novel or with a very rare minor allele frequency ( $\leq 0.0002$ ). Of these,  
154 p.Glu155Lys is present in EF-hand domain whereas p.Val316Ile, p.Gly433Ala and  
155 p.Arg509Pro are in prolyl 4-hydroxylase domain (**Table 1; Supplementary Figure 1**).

156 The seven mutation carriers presented severe, early-onset obesity between 0.3 to 3 years with  
157 a BMI SDS for age  $\geq 4$ . In addition to obesity, the carriers presented with hypotonia,  
158 developmental delay and cognitive impairment. In addition, a variable set of other



159 abnormalities was also observed in individual cases. Three of 5 probands from SOPP died  
160 from respiratory infection during the first ~2 years of the follow up (**Supplementary Figures**  
161 **1,2, Table 2**).

162 The prediction of the stability of the protein was carried out for four out of seven mutations  
163 where crystal structural information was available. The quantitative stability changes ( $\Delta\Delta G$ )  
164 upon mutations indicated a destabilizing effect and revealed an increase in flexibility  
165 (**Supplementary Table 3**). Through extensive molecular dynamics simulations, mutants  
166 showed fluctuations in both catalytic domains, EF hand (residues 190 - 290) domain, and the  
167 double-stranded  $\beta$ -helix (DSBH) fold (residues 310 - 460) (**Figure 2**). Overall, the structural  
168 analysis revealed domain movements compared to its *wt-P4HTM*. Details of the structural  
169 interpretation of mutations are provided in the **Supplementary Information**.

170 Finally, using ‘HypoMap’, an integrated reference atlas of the murine hypothalamus (14), we  
171 found that *P4HTM* is expressed ubiquitously in all regions of the hypothalamus, and in all  
172 cell-types, including neurons, oligodendrocytes, astrocytes and tanycytes (**Supplementary**  
173 **Figure 3**). Similar expression of P4HTM was observed in the hypothalamic cells from a  
174 single-nucleus sequencing dataset from adult human brains. This broad expression profile is  
175 consistent with the observed complex phenotype, beyond severe obesity, of the patients  
176 (**Supplementary Figure 5**). Non-fasting serum levels of metabolic hormones, leptin, insulin  
177 and cortisol, determined in the five affected patients from the SOPP were within the normal  
178 range except in one proband that presented hyperinsulinemia (Table 2).

179

180

## 181 **DISCUSSION**

182 We report seven paediatric patients with severe obesity carrying rare or novel bi-allelic  
183 missense mutations in *P4HTM*, representing a form of monogenic syndromic obesity,  
184 following a stepwise gene-centric analysis. The affected subjects were previously found  
185 negative for all known obesity genes. The phenotypes of all the affected subjects, in addition  
186 to obesity, included hypotonia, intellectual disability, and developmental delay. Importantly,  
187 these neurologic characteristics are consistent with the recently proposed HIDEA syndrome  
188 (acronym for **h**ypotonia, **i**ntellectual **d**isability, and **e**ye **a**bnormalities). This syndrome was  
189 first described in 2014 in a single Finnish family carrying pathogenic mutations in three genes  
190 - transketolase (*TKT*), prolyl 4-hydroxylase transmembrane (*P4HTM*), and ubiquitin specific  
191 peptidase 4 (*USP4*) (16). HIDEA syndrome was later suggested to be only caused by biallelic  
192 pathogenic mutations in *P4HTM*, and associates with hypotonia, hypoventilation, intellectual  
193 disability, dysautonomia, developmental delay and eye abnormalities (17). To date, 10  
194 families have been reported with homozygous or compound heterozygous mutations in  
195 *P4HTM* (16-19). Obesity phenotype was not emphasized in any of the reports, but our  
196 secondary assessment of the published phenotypes revealed that in 2 families, mutation  
197 carriers presented obesity (17; 19).

198 It is noteworthy that all *P4HTM* mutations found in the SOPP cohort are missense, which is  
199 also the case for the three previously published individuals with severe obesity, from the same  
200 family(19). On the contrary, all the other families showing severe neurological phenotypes  
201 that characterize HIDEA but lacking obesity (with the exception of one individual), have loss-  
202 of-function mutations (i.e. stop-gain, frame-shift and splice site). It is possible that this  
203 phenotypic heterogeneity reflects an allelic heterogeneity responsible for variable impairment  
204 of brain function, that affects or not the central control of appetite and/or energy balance.

205 *P4HTM* encodes the enzyme P4H-TM, an atypical member of the hypoxia inducible factors  
206 prolyl 4-hydroxylases (HIF-P4Hs) that are necessary for survival and adaptation under  
207 reduced oxygen supply and oxygen deprivation (20). Four members of the HIF-P4H family  
208 have been identified so far (21). In the scenario of normal oxygen supply - normoxia, the HIF-  
209 P4Hs get activated resulting in post-translational hydroxylation of HIF- $\alpha$  by converting  
210 prolines to 4-hydroxyprolines leading to its degradation. In the event when cells suffer from  
211 hypoxia, the HIF- $\alpha$  related hydroxylation reactions mediated by the P4Hs are suppressed  
212 resulting in HIF- $\alpha$ , accumulation and its dimerization with HIF- $\beta$  and migration into the  
213 nucleus for activation of several target genes responsible for maintaining oxygen homeostasis  
214 (22). Unlike other P4Hs members, P4H-TM is a transmembrane enzyme with a high degree of  
215 expression in the brain, predominantly in the hippocampus, amygdala and hypothalamic  
216 regions (23). However, its physiological function in the brain is yet unknown (24; 25). Our  
217 data suggest that genetic disruption of *P4HTM* also causes childhood obesity, possibly  
218 through dysregulation of appetite.

219 The very high mortality at a very young age of the children with *P4HTM* mutations, of the  
220 SOPP cohort (7 documented deaths including 3 probands and 4 family mutation carriers) is an  
221 ongoing tragedy. Furthermore, the deaths are apparently not related to central defects but  
222 possibly to lung hypoventilation following pneumonia and possibly associated with defective  
223 immunity (as reported in leptin deficient children).

224 Gene-centric analysis using MiST (mixed effects score tests) method was employed to detect  
225 this gene. This is an efficient approach specifically designed to prioritise rare variants by  
226 using a set-based score testing framework(26). This method uses a set of two score statistics  
227 derived by testing the group effect by variant characteristics and the heterogeneity effect  
228 making it a powerful and efficient approach. Previously, similar analysis used by us led to the

229 identification of a significant burden of pathogenic variants in genes linked with monogenic  
230 diabetes among patients clinically diagnosed with common type 2 diabetes (27).

231 In summary, pathogenic mutations in *P4HTM* cause, in the SOPP cohort, severe obesity  
232 associated with neurologic features of the HIDEA syndrome in 1.5% of our probands. It is not  
233 restricted to Pakistan as we identified two other patients from consanguineous families of  
234 Indian and Moroccan origin. The severity of this obesity associated syndrome with a high risk  
235 of mortality, highlights the advisability of screening *P4HTM* in young patients with severe  
236 obesity that also present (or express) hypotonia and intellectual disability/developmental  
237 delay, for a timely and appropriate management and care. Beyond linking *P4HTM* to obesity,  
238 we have demonstrated the power of stepwise statistical analysis using MiST followed by  
239 stringent filtration steps in bringing to surface new genes that are also associated with obesity.

240

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264

## 265 **AUTHORS CONTRIBUTION**

266 SS, AB, MA and FP designed the study and wrote the first draft of the paper. SS, RH, JM,  
267 WIK, LC, AT, AK, ISF, and MA recruited the samples. RH, QMJ and MA performed  
268 biochemical analysis. SS, A Badreddine, BT, EV, SA, MD and AB performed whole exome  
269 sequencing and analyzed the genetic data. LN and MB carried out statistical analysis. MUM  
270 and JFT carried out molecular modelling. ANM, BYH and GSHY performed expression  
271 analysis in murine hypothalamus. All authors contributed to the final version of the  
272 manuscript.

273

## 274 **CONFLICT OF INTEREST**

275 The authors have declared that no conflict of interest exists.



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381 **Table 1** Homozygous mutations identified in *P4HTM* (NM\_177938.2) among severely obese  
 382 children from consanguineous populations.

<b>ID</b>	<b>Mutation</b>	<b>PolyPhen</b>	<b>SIFT</b>	<b>Origin (study)</b>	<b>MAF overall (in GnomAD)</b>	<b>MAF SA in GnomAD)</b>
<b>Proband I</b>	c.1298G>C; p.Gly433Ala	Probably Damaging	Damaging	Pakistan (SOPP)	NA	NA
<b>Proband II</b>	c.946G>A; p.Val316Ile	Probably Damaging	Damaging	Pakistan (SOPP)	0.000003	0.000
<b>Proband III</b>	c.1526G>C; p.Arg509Pro	Probably Damaging	Damaging	Pakistan (SOPP)	NA	NA
<b>Proband IV</b>	c.889G>A; p.Val297Met	Probably Damaging	Damaging	Pakistan (SOPP)	0.00001	0.00013
<b>Proband V</b>	c.134C>T; p.Pro45Leu	Possibly Damaging	Damaging	Pakistan (SOPP)	0.0002	0.0021
<b>Proband VI</b>	c.347G>A; p.Gly116Asp	Probably Damaging	Damaging	Indian (GOOS)	0.000005	0.00003
<b>Proband VII</b>	c.463G>A; p.Glu155Lys	Probably Damaging	Damaging	Morocco	NA	NA

383 MAF SA: Minor Allele Frequency South Asian, NA: Not available.

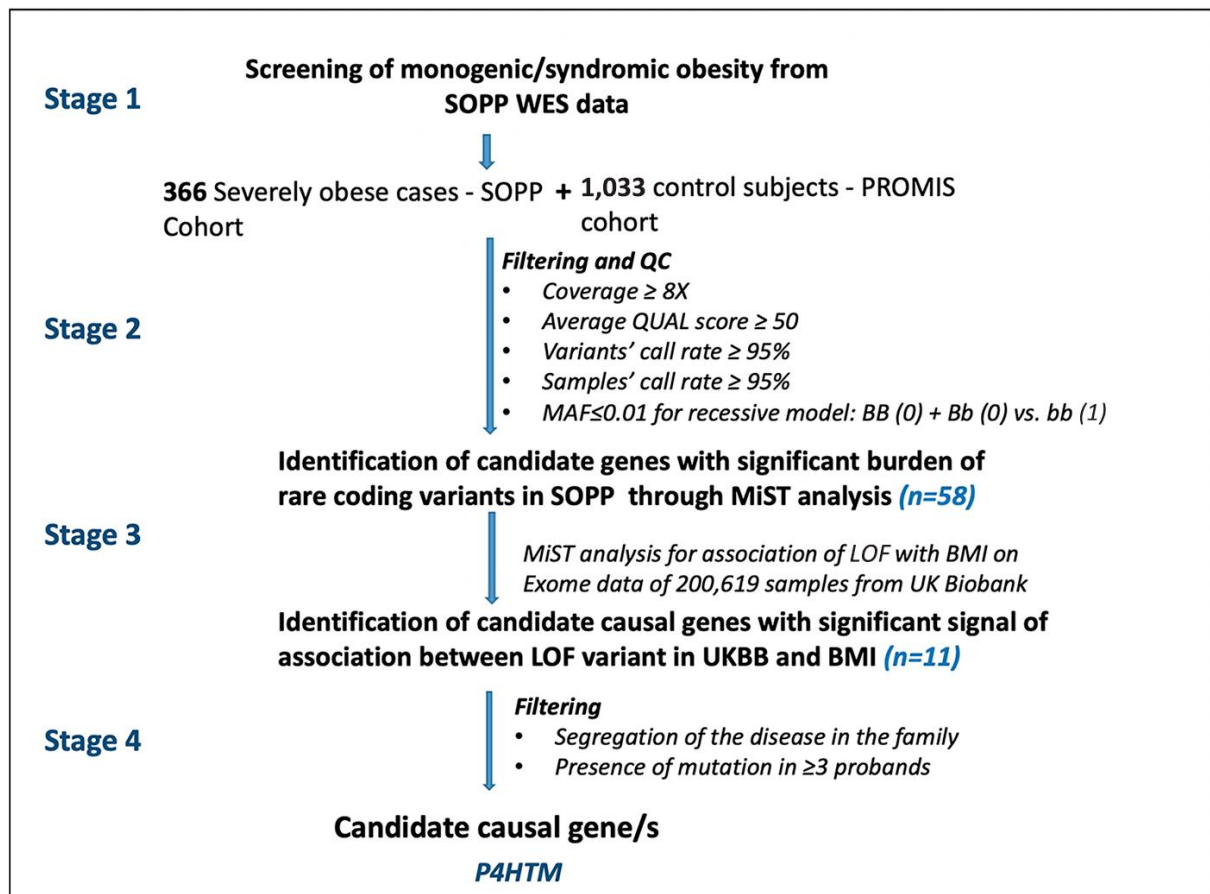
**Table 2** Clinical profile of severely obese children identified with balletic mutations in *P4HTM*.

ID	Proband I	Proband II	Proband III	Proband IV	Proband V	Proband VI	Proband VII
Age at first recruitment (Years)	4.5	0.9	5.0	3.9	9.6	3.0	4.0
Sex M/F	M	M	M	M	M	M	M
Age of obesity onset (Years)	0.9	0.3	1.0	0.6	3.0	NA	NA
SDS (BMI for age)	6.8	6.7	6.8	8.0	5.7	NA	NA
Alive/deceased	Deceased (5.2 years of age due to severe pneumonia)	Deceased (1.5 years of age due to pneumonia and high-grade fever)	Deceased (6 years of age; cause of death unknown)	Alive	Alive	Alive	Alive
Leptin (ng/ml)	3	18	10	16	12	NA	41.9
Insulin (uIU/ml)	26	19	122	5	12	NA	NA
Cortisol (ug/dl)	11	7	11	10	6	NA	NA
Intellectual disability	Yes	Yes	Yes	Yes	No	Yes	Yes
Hypotonia	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Deaths in family	The sisters at the age of 2 and 3.5 years and one cousin at age of 1.5 died as the result of kidney dysfunction (proteinuria)	His elder brother who shared the similar clinical phenotype died at the age of 2 years due to pneumonia	NR	NR	NR	NR	NR
Birth by caesarean section	Yes	Yes	No	Yes	No	NA	NA
Additional features	Polydipsia and polyuria, eye abnormalities including astigmatism, reported stubborn and aggressive in temperament	Suffered hepatomegaly, asthma and abdominal pain	Delayed milestones, aggressive behaviour, self-mutilation/self-harm, neonatal hypotonia, Thalassemia	Scoliosis, abnormal gait (needs support for walking, pneumonia twice but recovered, aggressive and stubborn	Asthma, breathing issue, sleep apnea	Epilepsy, hypoventilation, developmental and cognitive impairment, autonomic dysfunction	Hearing issues, cryptorchidism

F: Female; M: Male; NR: Not reported; SDS: Standard Deviation Score.

**Figure Legends:**

**Figure 1.** Schematic presentation of stepwise analyses and filtration strategy used in this study



**Figure 2. Structural interpretation of *P4HTM* missense variants.** **A)** The *P4HTM* structure is presented as a secondary structure topology, where the EF domain is shown in orange, the DSBH core in green, and the rest of the protein in yellow. **B)** Root-mean-square-deviation of mutants and wild-type (black) obtained over a period of 100 ns. The individual domains are mentioned, while iron-binding residues are highlighted in the yellow section. **C)** The MD simulated conformations (10 snapshots after every 20 ns) of mutants and wild type. Different regions of *P4HTM* are colored to increase the visibility of backbone deviations. **D)** The movement of domains (EF and DSBH) are displayed during MD simulations. **E)** Superimposition of MD simulated conformations of FG-2216 (sticks representation in distinct colour) in the active site of mutants and wild-type.  $\text{Fe}^{2+}$  is positioned inside the active site of *P4HTM* (molecular surface representation). **F)** Molecular mechanics generalized born surface area (MM-GBSA) binding free energies (kcal/mol) for mutants and wild-type/FG-2216 complex.

