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Identifying new potential genetic biomarkers for HELLP syndrome using massive parallel sequencing

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1 **Identifying new potential genetic biomarkers for HELLP syndrome**
2 **using massive parallel sequencing**

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Abstract

Background: Preeclampsia (PE) is a frequently occurring multisystemic disease affecting ~5% of pregnancies. PE patients may develop HELLP syndrome (haemolysis, elevated liver enzymes, and low platelet), a mother and foetus life-threatening condition. Research into HELLP's genetic origin has been relatively unsuccessful, mainly because normal placental function and blood pressure regulation involve the fine-regulation of hundreds of genes.

Objective: To identify new genes and mutations constituting potential biomarkers for HELLP syndrome.

Study design: The present case-control study involved whole-exome sequencing of 79 unrelated HELLP women. Candidate variants were screened in a control population constituted by 176 individuals. Stringent bioinformatics filters were used for selecting potentially etiological sequence variants in a subset of 487 genes. We used robust *in silico* mutation modelling for predicting the potential effect on protein structure.

Results: We identified numerous sequence variants in genes related to angiogenesis/coagulation/blood pressure regulation, cell differentiation/communication/adhesion, cell cycle and transcriptional gene regulation, extracellular matrix biology, lipid metabolism and immunological response. Five sequence variants generated premature stop codons in genes playing an essential role in placental physiology (*STOX1*, *PDGFD*, *IGF2*, *MMP1* and *DNAH11*). Six variants (ERAP1- p.Ile915Thr, ERAP2- p.Leu837Ser, COMT-p.His192Gln, CSAD-p.Pro418Ser, CDH1- p.Ala298Thr and CCR2-p.Met249Lys) led to destabilisation of protein structure as they had significant energy and residue interaction-related changes. We identified at

59 least two mutations in 57% of patients, arguing in favour of a polygenic origin for the
60 HELLP syndrome.

61 **Conclusion:** Our results provide novel evidence regarding PE/HELLP's genetic origin,
62 leading to new biomarkers, having potential clinical usefulness, being proposed.

63

64 **Key words:** Genetic biomarkers; HELLP syndrome; molecular medicine; protein
65 modelling; whole-exome sequencing (WES).

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84 **Introduction**

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86 Preeclampsia (PE), clinically characterised by pregnancy-induced hypertension and
87 proteinuria after the 20th week of gestation, is a frequently occurring multisystemic
88 disease affecting ~5% of pregnancies [1,2]. Women affected by PE can suffer foetal
89 intra-uterine growth restriction (IUGR) and have an increased risk of being affected by
90 cardiovascular disease later in life [3,4]. It has been seen that ~15% of patients affected
91 by severe PE may develop HELLP (haemolysis, elevated liver enzymes, and low
92 platelet) syndrome, a mother and foetus life-threatening condition. Concerning PE *per*
93 *se*, mechanisms have been described regarding PE, such as defective deep placentation,
94 anticoagulant factor activation, immune response at the placental–maternal interface,
95 increased endoplasmic and oxidative stress, having autoantibodies to type-1 angiotensin
96 II receptor, intravascular inflammation, angiogenesis imbalance and endothelial
97 dysfunction [5].

98 However, the exact mechanisms triggering PE developing into HELLP syndrome have
99 not been completely elucidated. Several potentially PE-linked biochemical markers (e.g.
100 PAPP-A, free CG- β , AFP, PP13, ADAM12, PIGF, VEGF, P-Selectin and inhibin A)
101 have been studied for identifying those underlying possible usefulness in clinical
102 environments [5]. For the moment, while some of these markers are indeed associated
103 with preeclampsia, the effectiveness of those for predicting early PE is still disputable.
104 Regarding genetics of PE, at least 15 PE loci have been mapped to date and candidate
105 genes have been proposed, such as *ACVR2A*, *TNFSF13B*, *EPAS1* and *STOX1*[6,7]. The
106 *STOX1* c.457T>C (p.Tyr153His) mutation has been functionally linked to PE aetiology,
107 while *Stox1* overexpression in mice leads to PE, IUGR and cardiovascular disease [8–12].
108 The p.Ala356Gly deleterious mutation in Forkhead Box D1 (FOXD1), leading to C3

109 promoter transactivation disturbances, has been identified recently in a woman suffering
110 PE and IUGR [13,14]. Some sequence variants in other genes (i.e. *VEGF*, *MMP9*,
111 *SERPINEA8* and *MMP9*) seem to increase the risk of PE.

112 Regarding the HELLP syndrome, a few genetic studies have been undertaken and it has
113 generally been accepted that, due to common molecular pathways, most PE-related genes
114 may also contribute towards HELLP's aetiology.

115 However, research into PE/HELLP's genetic origin has been relatively unsuccessful,
116 mainly because normal placental function and blood pressure regulation involve the fine-
117 regulation of hundreds of genes. Furthermore, large pedigrees displaying PE/HELLP are
118 rare, thereby generally hampering the mapping of genomic regions by classic genetic
119 approaches. Whole-exome sequencing (WES) has been carried out in familial and
120 isolated cases of PE which led to proposing some possible new genes and sequence
121 variants related to the phenotype [15–18]. However, no large-scale genomic approaches
122 have yet been adopted in patients with HELLP syndrome.

123
124 Here is presented the first WES study for patients affected by the HELLP syndrome, on a
125 relatively large cohort (79 unrelated women) of Colombian origin. Stringent
126 bioinformatics filters were used for selecting potentially etiological non-synonymous
127 sequence variants in a subset of 487 genes. We used robust *in silico* mutation modelling
128 for predicting the potential effect on protein structure. We identified 110 variants (87
129 different) in 67 genes related to several biological processes, such as
130 angiogenesis/coagulation/blood pressure regulation, cell
131 differentiation/communication/adhesion, cell cycle and transcriptional gene regulation,
132 extracellular matrix biology, lipid metabolism and immunological response. Five
133 sequence variants were particularly drastic as they generated premature stop codons in

134 genes playing an essential role in placental physiology (e.g. STOX1, PDGFD, IGF2,
135 MMP1 and DNAH11). Six variants (ERAP1-p.Ile915Thr, ERAP2- p.Leu837Ser,
136 COMT-p.His192Gln, CSAD-p.Pro418Ser, CDH1-p.Ala298Thr and CCR2-p.Met249Lys)
137 led to potential destabilisation of protein structure as they had significant energy and
138 residue interaction-related changes. We identified at least two mutations in 57% of
139 patients, arguing in favour of a polygenic origin for the HELLP syndrome.

140
141 Taken together, our results provide novel evidence regarding PE/HELLP's genetic origin,
142 leading to new biomarkers, having potential clinical usefulness, being proposed.

143
144 **Materials and Methods**

145
146 *Women affected by HELLP syndrome and control population*

147 The study involved selecting 79 unrelated women affected by HELLP syndrome for WES
148 sequencing. The patients (all from Colombia) are part of the GenPE study (Genetics and
149 Pre-eclampsia), large, multi-centre, case-control study conducted between December
150 2000 and February 2012 in eight Colombian cities (www.genpe.org) or the Hospital
151 Universitario Mayor-Méderi (Bogotá, Colombia). The **Supplementary Table 1** includes
152 the clinical characteristics of the HELLP patients.

153 To calculate the sample size, we have first explored a Colombian health database (named
154 SIVIGILA) that records numerous epidemiological events
155 (<http://www.ins.gov.co/buscador>). We found that 22,915 women were affected by
156 extreme gestational morbidity, 44.2% of which were suffered severe preeclampsia (PE).
157 Thereafter, based on the reported frequency of HELLP (~20%) we estimated that 2028
158 should be affected by the disease. This number was taken as the total population. The

159 final calculation (5% proportion), which was performed considering a precision of 5%
160 and a confidence interval of 95%, led us to establish a sample size of 70 patients.

161 The Tennessee Classification System diagnostic criteria for HELLP syndrome [19] were
162 used for selecting patients. The main diagnostic laboratory criteria were thus: \leq
163 100,000/L platelets, \geq 70 IU/ AST L and \geq 600 IU/L LDH. This classification system
164 rates HELLP syndrome as being complete (three criteria) or incomplete (one or two
165 criteria). Candidate variants were screened using WES data from 176 Colombian women
166 (control group, C) over 50 years old having at least one child and lacking antecedents of
167 medical complications during pregnancy. Individuals having antecedents of hypertensive
168 disorders were excluded as controls. The Universidad del Rosario (Code: DVN-021-1-
169 063, May 2017) Ethics' Committee approved the clinical study and all experimental
170 procedures. Clinical investigation followed Declaration of Helsinki guidelines (1975, as
171 revised in 1996). All individuals had signed informed consent forms.

172

173 *WES, bioinformatics filtering of variants, Sanger sequencing and interactome analysis*

174 Genomic DNA was extracted from all patients' blood samples, using standard
175 procedures. Details regarding NGS experiments and primary bioinformatics analysis have
176 been included as **Supplementary methods**. The detected sequence variants were filtered
177 in parallel using R (v3.5.1; www.r-project.org), Excel (Microsoft Office 365) and VarSeq
178 (Golden Helix Inc., Bozeman, USA) software. A subset of 487 candidate genes (named
179 HELLP-487) was created by screening several databases and used to filter rare (MAF
180 <0.005) variants evoking potentially harmful effects (**Supplementary methods,**
181 **Supplementary Table 2**). **Figure 1** illustrates the bioinformatics pipeline used for
182 candidate variants' filtering. We screened these variants in the C group of women. All
183 selected variants were verified (n=118) by Sanger sequencing. The primer sequences and

184 PCR sconditions are available upon request. We performed a network analysis of the
185 genes where variants have been found, build using String-db (<https://string-db.org/cgi/>).
186 A functional enrichment analysis was carried out using Webgestalt against the GLAD4U
187 disease database.

188

189 ***In silico* structure prediction of protein mutant forms**

190 The structural biology function of the DNASTAR Lasergene Suite software was used for
191 predicting *in silico* energy/structure disturbances (ΔE -DFIRE-A calculations) induced by
192 amino acid (aa) changes [20]. Details concerning these experiments have been included
193 as **Supplementary Method**.

194

195 **Results**

196

197 ***Sequencing and bioinformatics analysis***

198 WES experiments' genomic coverage was higher than 98% on average. More than >90%
199 of the genome was sequenced in all samples, having an average 50X read depth.
200 25,912,887 sequence variants were found in the 79 patients affected by the HELLP
201 syndrome, 1,272,558 of which were non-synonymous or affected splicing sites (**Figure**
202 **2**). It was found that 277,570 variants had ≤ 0.005 MAF; 2,253 of these occurred in the
203 HELLP-487 gene subset. Implementing computational predictors gave 119 variants
204 having scores compatible with potential deleterious effects. Thereafter, the *DYSF*
205 c.3287G>A variant was discarded as it had >0.005 MAF in the C group. Sanger
206 sequencing confirmed 110 out of 118 (93%) filtered variants (**Table 1 and Figure 2**).
207 One hundred and six and four of these were at heterozygous and homozygous state,
208 respectively (**Table 1**). We identified 87 different sequence variants of different types: 60

209 missense, 21 indels, 5 nonsense and 1 splice-site. The **Supplementary Figure 1** shows
210 the results of the STRING interactome analysis. The network probability for
211 enrichment was $p=1.18 \times 10^{-5}$. The gene ontology analysis revealed in particular KEGG
212 and Reactome pathways linked to the coagulation cascade (FGG, F5, SERPINC1,
213 SERPINB2). The analysis by Webgestalt against the GLAD4U disease database revealed
214 the significant enrichments (FDR <0.05), also focused on thrombosis defects
215 (**Supplementary Figure 2**).

216

217 *Structural modelling of mutant proteins*

218 Various protein structures were identified in the PDB database which facilitated
219 modelling mutations identified in patients suffering HELLP syndrome (**Table 2**). Using P
220 models (n=12) for simulating the prediction algorithm revealed that only 8% of the
221 polymorphic changes modified a protein's local physicochemical properties
222 (**Supplementary Table 3**). Ten out of the 17 candidate mutants (57%) found in patients
223 affected by HELLP (for which PDB structures were available for *in silico* modelling) had
224 significant changes regarding ΔE and residue interactions (**Supplementary Table 3**). Six
225 of them (ERAP1- p.Ile915Thr, ERAP2- p.Leu837Ser, COMT-p.His192Gln, CSAD-
226 p.Pro418Ser, CDH1- p.Ala298Thr and CCR2-p.Met249Lys) led to destabilising protein
227 structure (**Figures 3, 4 and 5, Table 2, Supplementary Figures 3, 4 and 5**).

228

229 **Discussion**

230 The present study focused on 79 women affected by an extreme hypertensive-related
231 phenotype to optimise the chances of discovering sequence variants potentially
232 contributing to the disease's pathogenesis. Studying extreme phenotypes via NGS has
233 been associated with an ability for identifying causal rare variants participating to

234 complex traits [21–24]. It is known that rare encoding variants conferring
235 moderate/strong functional effects significantly contribute towards frequently occurring
236 diseases' phenotype heritability [25–27]. Our analysis involved opting for creating an
237 extensive subset of 487 candidate genes having molecular relevance in placental
238 development/function and preeclampsia/HELLP pathophysiology. We, and other groups,
239 have demonstrated that this strategy provides an efficient alternative for dissecting
240 complex molecular cascades involved in frequent reproductive disorders, such as
241 recurrent spontaneous abortion and primary ovarian insufficiency [28–31]. Interestingly,
242 using computational filters in those studies (similar to those used in the present work)
243 enabled identifying mutations leading to significant structural/functional disturbances,
244 several of which were validated thereafter by *in vitro* assays [32,33].

245 To identify candidate variants we used specific astringent filters (non-synonymous
246 variants, <0.005 MAF, four bioinformatics predictors of deleterious effects, protein
247 sequence alignments). The genomAD database was implemented as a robust source of
248 genomic information (> 60,000 control individuals). The very low frequency of these
249 variants in the database argued in favor of a potential causative effect. We selected 119
250 sequence variants, 110 of which were definitely confirmed by Sanger sequencing (92%).
251 This corroborated our NGS experiments' technical reliability. We screened these variants
252 in a cohort of 176 individuals having the same ethnic origin, which led us to discarding
253 one of them having MAF >0.005. In genetic studies, large number of controls is useful to
254 establish, from an epidemiological perspective, a robust causative link between candidate
255 variants and the disease aetiology. This is particularly true for genetic association studies
256 (e.g. GWAS) in which thousands of controls are necessary to map frequent variants
257 potentially related to the phenotype. In GWAS, which is based on unbiased genetic
258 hypotheses, the candidate variants generally underline low-to-moderate functional effects

259 and/or constitute only mapping genomic markers. For sure, during almost two decades
260 this approach led to map hundreds of chromosomal regions and genes contributing to
261 complex diseases. The present approach was slightly different, because we analyzed
262 exclusively the coding regions of a gene subset and performed a very stringent
263 bioinformatic variant filtering. This strategy allows determining variants underlying
264 moderate-to-severe functional effects, which are rare in the general healthy population as
265 they tend to be negatively selected.

266 We found that the affected genes were related to several biological processes, such as
267 angiogenesis/coagulation/blood pressure regulation, cell
268 differentiation/communication/adhesion, cell cycle and gene regulation, extracellular
269 matrix biology, immunological process and lipid metabolism. These genes may
270 participate in various biological processes simultaneously; however, we have classified
271 each one into just a single category for simplicity.

272 ERAP1 and ERAP2 are two reticulum endopeptidases participating in the
273 processing/regulation of important hormones (e.g. angiotensin) and other molecules
274 (nitric oxide, VEGF) involved in modulating blood pressure, angiogenesis and
275 inflammation [34–38]. For instance, the ERAP1 p.Lys528Arg polymorphism was
276 associated with essential hypertension; ERAP2 expression disturbances in placenta, as
277 well ERAP1/ERAP2 sequence variants, were linked to an increased risk of suffering PE
278 [39–43]. The p.Lys528Arg mutation, located in the catalytic domain, has been shown to
279 reduce protein efficiency to cleave angiotensin II (to angiotensin III) and to transform
280 kallidin into bradykinin [35].

281 The ERAP1-p.Ile915Thr mutation, identified in Pt-88, is located in the protein's C-
282 terminal region, in a bowl-shaped alpha-helix subdomain (aa 614–941), consisting of
283 numerous α -helices forming a concave surface completely sealing off the active site [44–

284 47]. This protein region (domain IV), which has been highly conserved during evolution
285 and among other enzyme subfamily members, plays an essential role as a “molecular
286 ruler” mechanism, i.e. allosteric activation which involves antigenic peptide recognition
287 and attachment. Interestingly, it has been proposed that the ERAP1 C-terminal region’s
288 hydrophobic pocket could trim the angiotensin II peptide, underlining a relevant role in
289 blood pressure regulation [47]. Structural *in silico* modelling revealed that the ERAP1-
290 p.Ile915Thr mutant form led to a significant change in residue interaction energies
291 ($\Delta E=1.708$), which was related to destabilising protein structure (**Figure 3, Table 2**).
292 Furthermore, the Ile⁹¹⁵ aa interacted with 12 residues in the α -helix in ERAP1-WT
293 structure, whilst Thr⁹¹⁵ interacted with 11 aa in the mutant form (the Thr⁹¹⁵-Val⁸⁹¹
294 interaction became lost). The p.Ile915Thr mutation might therefore affect the C-terminal
295 domain’s folding, leading to ERAP1 enzymatic dysfunction, thereby contributing to the
296 patient’s phenotype.

297 Regarding ERAP2, the p.Leu837Ser mutation is also located in the IV domain
298 (encompassing residues 648-960) and might also disturb catalytic site capping [48]. This
299 variant led to a strong interaction energy disturbance ($\Delta E=2.414$) which was also
300 predicted to destabilise protein structure (**Figure 4, Table 2**). Furthermore, we
301 ascertained that the mutant Ser⁸³⁷ residue lost interactions with 3 residues (Leu⁹²⁸, Ser⁸²⁹,
302 Lys⁸⁶⁷) in the α -helix, compared to its Leu⁸³⁷ WT counterpart. This variant occurred in
303 two patients in homozygous state but was absent in the C group, thereby arguing in
304 favour of a specific allele which occurs frequently in the Colombian population. The
305 ERAP2- p.Pro548Leu mutation is located at the junction region between domain II
306 (residues 272-546), containing the HEXXH₁₈E zinc-binding motif and the
307 aminopeptidase motif, and hinge region domain III. It is worth noting that this variant had
308 a change ($\Delta E=-2,023$) which was predicted to stabilise protein structure; however, due to

309 the arguments given above, a functional deleterious effects is still likely. Indeed, it should
310 be stressed that predicting single variants' potentially deleterious effects should result
311 from studying different variables (genetic variability, evolutionary conservation, protein
312 structural properties, functional effects and interactome impacts). Local/global structural
313 changes in the protein triggered by *ERAP1* and *ERAP2* sequence variants found in our
314 patients affected by HELLP syndrome might therefore alter enzymatic activity and
315 substrate cleavage.

316
317 The *COMT* gene encodes the catechol-O-methyltransferase enzyme that metabolises
318 estradiol. COMT is involved in maintaining oxygen balance in placenta via HIF-1a/FLT1
319 (VEGFR) regulation, which has led to proposing it as a relevant molecular actor in
320 preeclampsia pathophysiology [6]. Low COMT levels in humans have been observed in
321 women affected by severe PE, whilst the homozygous *Comt* gene KO in mice has
322 induced a preeclampsia-like syndrome [49,50]. Functional p.Val158Met polymorphism
323 has been associated with PE susceptibility in several populations, especially in
324 homozygous carriers [51].

325 Interestingly, it has been stated that His¹⁹² residue replacement by a Gln (as we found in
326 Pt-72) plays a central role in the protein's active site functioning, since it binds the S-
327 adenosylmethionine substrate methyl donor [52]. Structural analysis of the mutant protein
328 revealed an $\Delta E=0.726$ energetic change (destabilising effect) which was related to the
329 loss of at least two residues' interactions (His¹⁹²- Asp²⁰⁰ and His¹⁹²-Arg¹⁹⁶). Moreover,
330 we observed a new interaction between mutant Gln¹⁹² and Ile¹⁴¹ (**Figure 5**). We
331 hypothesise that this mutation might contribute to dysfunction occurrence, similarly to
332 that observed in *Comt*^{-/-} pre-eclamptic-like mice which had placental vascular lesions
333 (e.g. acute atherosclerosis), an absence of 2-methoxyoestradiol (2-ME) and hypersensitivity to

334 angiotensin II [49,53]. It is worth noting that we identified further destabilising changes
335 in 3 proteins in the present work: CSAD (p.Pro418Ser, $\Delta E=0.654$), CDH1 (p.Ala298Thr,
336 $\Delta E=0.641$) and CCR2 (p.Met249Lys, $\Delta E=2.335$) (**Supplementary Figures. 3, 4, 5,**
337 **Table 2**). These factors have not yet been extensively studied in PE/HELLP and therefore
338 constitute coherent candidates for future studies.

339 Interestingly, regarding proteins involved in cell cycle and transcriptional gene
340 regulation, we found variants in 5 transcription factors (TFs): STOX1, STOX2, CEBPA,
341 TBX15 and DLX6 TFs. Previous reports have proposed that particular sets of TFs might
342 be partly responsible for the massive transcriptomic dysregulation of genes observed in
343 pre-eclamptic placentas [54]. Amongst these, *STOX1* was of particular interest as it is a
344 major actor involved in different cell processes involved in placental biology, such as
345 proliferation, migration/invasion, oxidative stress equilibrium regulation, and the
346 modulation of coagulation, inflammation and apoptosis [10]. Mutant forms of STOX1,
347 such as transgenic mice, cell models and the human Tyr153His sequence variant, have
348 been linked to PE [8,11,55,56].

349 STOX1 mutant forms' harmful effect arises from its overexpression or increased
350 transcriptional efficiency regarding particular targets.

351 The STOX1-p.Arg364Ter sequence variant was identified in one patient in the present
352 work and p.Thr188Asn in another four. Structurally, the p.Arg364Ter has a particularly
353 drastic effect as it is predicted to be slightly longer than the 'natural' STOX1B isoform
354 (227 amino acids), but much shorter than the STOX1A isoform (989 amino acids). Since
355 this mutant form encompasses the DNA binding domain and that in trophoblast STOX1
356 and STOX1B are generated about at the same concentration, it can be assumed that the
357 existence of the mutant allele will decrease the level of STOX1A to half the normal dose
358 leading to an increase of the STOX1B-like form. We recently showed that STOX1B

359 overexpression induces major defects in the syncytialization process of trophoblast cells
360 [57]. This mutation will therefore induce a moderate imbalance of STOX1A and B that
361 would presumably impact placental function and be associated to HELLP syndrome
362 onset.

363
364 The p.Thr188Asn variant is located in the DNA binding domain, potentially altering
365 target gene binding and transactivation properties. We thus hypothesised that subtle
366 equilibrium regarding STOX1 structure/expression/function is essential for placental
367 physiology and to avoid/escape PE/HELLP. Different types of STOX1 mutations might
368 lead to PE/HELLP *via* various molecular and functional overlapping mechanisms, some
369 of which remain unexplored.

370
371 Four additional sequence variants leading to premature stop codons were identified in
372 genes participating in different biological processes: *PDGFD*-c.1108C>T (p.Arg370Ter),
373 *IGF2*-c.97C>T (p.Gln33Ter), *MMP1*-c.1389G>A (p.Trp463Ter) and *DNAH11*-
374 c.4060G>T (p.Glu1354Ter). The *PDGFD*-p.Arg370Ter affects the C-terminal region of
375 platelet-derived growth factor D, a protein having diverse functions, including blood
376 vessel maturation during angiogenesis [58]. Although the p.Arg370Ter mutation affected
377 the protein's last residue, it highlighted a very likely harmful effect since Arg³⁷⁰ plays a
378 fundamental role in *PDGFD* binding to neuropilin 1 (NRP1); NRP1, in turn, is an
379 important co-receptor for VEGF family members, including the placental growth factor
380 (PlGF) [59,60].

381 The *IGF2*-c.97C>T (p.Gln33Ter) mutation in Pt-21 affects insulin-like growth factor-2
382 (*IGF2*), a maternal imprinted gene involved in placentation and foetal growth [61]. Some
383 heterozygous sequence variants (paternally inherited) in *IGF2* and epigenetic

384 modifications in the *IGF2/H19* locus have been reported in patients affected by Silver-
385 Russell syndrome (and Silver-Russell-like phenotypes). This disease is characterised by
386 intrauterine growth retardation, placental abnormalities, postnatal growth deficiency and
387 characteristic triangular facies [62–65]. The *IGF2-c.97C>T* (p.Gln33Ter) in Pt-21 has
388 been predicted to generate an early stop codon and a protein lacking IGF insulin receptor
389 binding sites. Since the premature stop codon is in close proximity to the start ATG
390 codon (e.g. <200 nucleotides), it is highly likely that the nonsense-mediated mRNA
391 decay system targets and degrades the mutant transcript [66]. Since *IGF2* is maternally
392 imprinted in the placenta, a possible explanation for its potential involvement is that the
393 nonsense variant in HELLP syndrome may cause uniparental disomy (UPD). Although
394 maternal UPD for chromosome 7 has been related to Silver-Russell syndrome, this
395 variant's association with the disease is speculative, as we do not know whether Pt-21 is
396 affected by any phenotype.

397
398 *MMP1-c.1389G>A* (p.Trp463Ter) was identified in Pt-11; this sequence variant affects
399 the gene encoding matrix metalloproteinase 1 (MMP1), a collagenase acting in
400 extracellular matrix molecule degradation and turnover in numerous biological processes.
401 MMP-1 plays an important role in PE pathogenesis as it participates in neutrophil
402 recruitment, reactive oxygen species (ROS) generation and stimulating vascular reactivity
403 to angiotensin II via various molecular pathways [67–69]. The *MMP1-p.Trp463Ter*
404 mutant protein lacks a C-terminal, which is contiguously located in the hemopexin-like
405 domain, a region required for the catalytic domain's proper functioning [70,71]. This
406 mutant may shape changes in the protein's conformational properties, thus contributing to
407 the phenotype. A nonsense heterozygous mutation in the *DNAH11* gene was identified in
408 Pt-98 and predicted to generate a truncated 1,354 residue-long protein (p.Glu1354Ter).

409 *DNAH11* homozygous mutations have been described as being causative of Kartagener
410 syndrome, a rare recessive disease lacking placental dysfunction. Although the *DNAH11*
411 mutation is drastic, it is difficult to establish its association with PE/HELLP.

412 Interestingly, 57% of the patients had at least two sequence variants, arguing in favour of
413 the HELLP syndrome's polygenic nature. Most sequence variants found in our work *via*
414 WES, using maternal DNA, were in heterozygous state. Since segregation analysis did
415 not form part of our work, we could not establish accurately which variants occurred in
416 placental (foetal) DNA, thereby affecting expression/function. Caution is thus advised
417 when interpreting some of our results.

418
419 To date, two WES studies on DNA samples from non-related PE women have been
420 published. Gammill et al. analysed specific genes, related to cardiomyopathy, in a 181
421 population of white PE patients. Mutations in genes contributing to idiopathic and
422 peripartum cardiomyopathy were found to be potentially associated with PE [72]. In
423 another study, Hansen et al. performed WES experiments on 50 Danish PE women and
424 50 controls for identifying candidate variants. Although two gene subsets were created
425 for downstream analysis, the authors were unable to describe strong candidates related to
426 the disease's aetiology [17]. These findings evoke the difficulty to map genetic
427 determinants and indicate that further NGS studies are necessary to identify new
428 genes/mutations contributing to PE/HELLP.

429
430 The results presented here cannot be generalized to other populations and have to be
431 analysed with caution. Undoubtedly, future genomic studies involving HELLP patients
432 from different ethnic origins are therefore mandatory to determine whether the genes and
433 mutations described by us are also relevant in other populations. The present findings are

434 descriptive but have a relevant significance concerning research into the genetic causes of
435 preeclampsia and HELLP syndrome and should constitute the starting point for new
436 research projects.

437 After sequence variants' functional validation, our findings have applications in clinical
438 practice, as they should help to the disease detection and prognosis.

439 The present results constitute the starting point for future investigations interested to
440 dissect, at molecular level, the inherent complexity of PE/HELLP phenotype. Future
441 studies should involve functional *in vitro* tests of candidate mutations and genetic
442 screening of variants in large panels of PE/HELLP patients.

443

444 **Conclusions**

445 Taken together, our results indicated that using large-scale genomic approaches for
446 patients having extreme phenotypes represents an efficient strategy for identifying the
447 genetic determinants potentially involved in complex diseases. HELLP syndrome
448 represent a frequent polygenic disease that can be studied efficiently by NGS technique.
449 The present findings add insights regarding HELLP syndrome's molecular origin and
450 propose a significant amount of new genes and mutations, which could be proposed as
451 new clinically useful molecular biomarkers.

452

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458

459 **Author contribution:**

460 SF, SB, ADA, TM, JA and NS performed the clinical study. KMJ, AM, LP, LFC, MAG,
461 DB and CC performed in silico analyses and molecular biology experiments. DV
462 analyzed the data and corrected the manuscript. PL designed and directed the study, and
463 wrote the manuscript draft. All authors read and approved the final version of the paper.

464

465 **Declaration of Competing interest:** The authors reports no conflict of interest.

466

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Table 1. Gene variants identified in women affected by HELLP syndrome.

Gene	ID	Variant		Zygoty	MAF	rs	Biological Process
		DNA	Protein				
<i>COMT</i>	72	c.576C>G	p.His192Gln	Hh	ND	ND	Angiogenesis/ Coagulation/ Blood pressure regulation
<i>DBH</i>	65	c.263G>C	p.Gly88Ala	Hh	0,0028	rs3025380	
<i>ENG</i>	25	c.179C>A	p.Ala60Asp	Hh	0,0004	rs146100407	
<i>ERAP1</i>	7	c.2744T>C	p.Ile915Thr	Hh	0,0008	rs139576768	
<i>ERAP2</i>	88	c.1643C>T	p.Pro548Leu	Hh	0,0001	ND	
	94	c.2510T>C	p.Leu837Ser	HH	0,0031	rs142362923	
	36	c.2510T>C	p.Leu837Ser	HH	0,0031	rs142362923	
<i>F5</i>	40	c.5460G>A	p.Met1792Ile*	Hh	0,0027	rs6026	
	46	c.5460G>A	p.Met1792Ile*	Hh	0,0027	rs6026	
<i>FGG</i>	51 m	c.571G>A	p.Gly191Arg	Hh	0,0026	rs6063	
<i>INHBB</i>	7	c.938G>A	p.Arg313His	Hh	ND	rs1317023671	
<i>KDM1A</i>	66	c.27_53del	p.Ala10_Ala18del	Hh	0,0001	rs762119456	
<i>KDR</i>	78	c.2312C>T	p.Thr771Met	Hh	0,0011	rs149745504	
<i>MAS1</i>	14	c.755A>G	p.Tyr252Cys	Hh	0,0025	rs147267209	
<i>PDGFD</i>	19	c.1108C>T	p.Arg370Ter	Hh	0,0002	rs550016351	
<i>SERPINB 2</i>	80	c.1111G>A	p.Ala371Thr	Hh	0,0010	rs139893427	
<i>SERPINC 1</i>	27	c.1246G>T	p.Ala416Ser	Hh	0,0009	rs121909548	
	90	c.749C>A	p.Thr250Asn	Hh	ND	ND	
<i>ADD1</i>	17	c.98C>T	p.Pro33Leu	Hh	0,0001	rs749350133	Cell differentiation/ Communication/Adhesi on
<i>CALCRL</i>	39	c.665T>G	p.Leu222Arg	Hh	ND	ND	
<i>CLDN6</i>	34	c.62delG	p.Gly21fs	Hh	ND	ND	
<i>CTNNAL1</i>	37	c. 719_720del	p.Thr240fs	Hh	ND	ND	
<i>CUL7</i>	87	c.3490C>T	p.Arg1164Trp	Hh	0,0021	rs201135654	
<i>DYSF</i>	84	c.3283C>T	p.Arg1095Cys	Hh	0,0018	rs141536854	
	7	c.3283C>T	p.Arg1095Cys	Hh	0,0018	rs141536854	
	8	c.3283C>T	p.Arg1095Cys	Hh	0,0018	rs141536854	
	97	c.707A>C	p.Asn236Thr	Hh	0,0001	rs150917600	
<i>HTR3A</i>	65	c.1096C>T	p.Arg366Trp	Hh	0,0001	rs147921110	

<i>PTGS1</i>	37	c.211C>T	p.Pro71Ser	Hh	0,0007	rs143357990	
	64	c.211C>T	p.Pro71Ser	Hh	0,0007	rs143357990	
	76	c.211C>T	p.Pro71Ser	Hh	0,0007	rs143357990	
	88	c.232C>T	p.Arg78Trp	Hh	0,0028	rs181947887	
<i>SELE</i>	16 m	c.763T>C	p.Cys255Arg	Hh	ND	ND	
<i>SELP</i>	22	c.2003G>A	p.Cys668Tyr	Hh	ND	ND	
<i>THBS4</i>	51	c.2001_2003dup	p.Asp668dup	Hh	0,0001	rs756728794	
<i>TNF</i>	29	c.130G>A	p.Ala44Thr	Hh	0,0000	rs867435711	
<i>APC</i>	40	c.3468_3470del	p.Glu1157del	Hh	0,0013	rs386833391	
<i>AR</i>	61	c.1186G>C	p.Gly396Arg	Hh	0,0001	rs202150225	
<i>CDH1</i>	86	c.892G>A	p.Ala298Thr	Hh	0,0005	rs142822590	
<i>CDKN1C</i>	3	c.573_590delCCCGGCT CCAGTCGCGGC	p.Ala191_Ala196del	Hh	ND	ND	Cell cycle and Transcriptional gene regulation
<i>CEBPA</i>	24	c.418_419insGCGGCG	p.Leu139_Asp140insGly	Hh	ND	ND	
<i>DLX6</i>	7	c.92_93insACAGCAGCA GCA	p.Gln41_Gln44dup	Hh	0,0002	rs761321946	
	39	c.92_93insACAGCAGCA GCA	p.Gln41_Gln44dup	Hh	0,0002	rs761321946	
	61	c.92_93insACAGCAGCA GCA	p.Gln41_Gln44dup	Hh	0,0002	rs761321946	
	80	c.92_93insACAGCAGCA GCA	p.Gln41_Gln44dup	Hh	0,0002	rs761321946	
	21 m	c.99_119del	p.Gln38_Gln44del	Hh	0,0002	rs762924495	
<i>EP300</i>	6	c.2091T>G	p.Ser697Arg	Hh	0,0034	rs61756764	
	28 m	c.6798_6800del	p.Gln2268del	Hh	0,0016	rs533875300	
<i>IGF2</i>	21	c.97C>T	p.Gln33Ter	Hh	0,0008	rs200441006	
<i>INO80B</i>	93	c.807_812dup	p.Ala270_Pro271dup	Hh	0,0002	rs764791821	
<i>MKI67</i>	24	c.4350_4351insCCCAGC	p.Gly1450_Ser1451ins ProSer	Hh	0,0030	rs139382420	

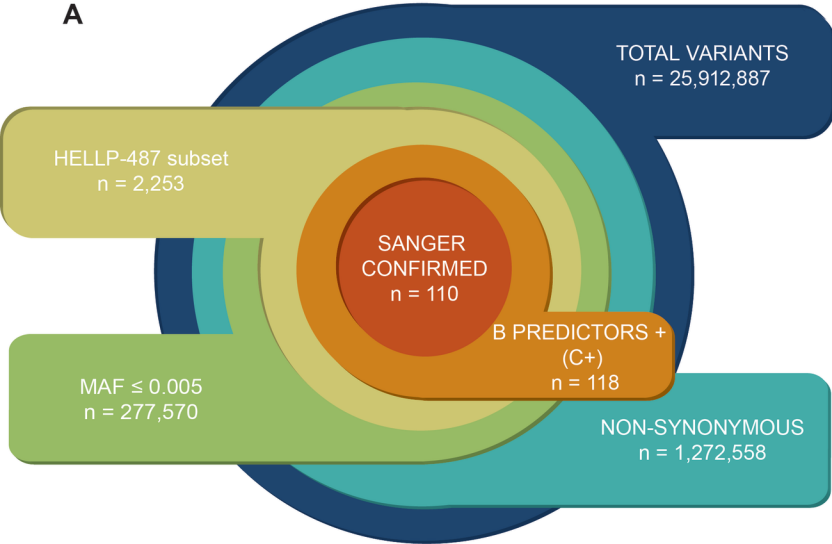
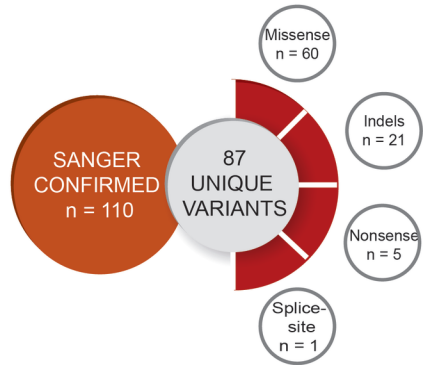
	97	c.4350_4351insCCCAGC	p.Gly1450_Ser1451ins ProSer	HH	0,0030	rs139382420		
	99	c.4350_4351insCCCAGC	p.Gly1450_Ser1451ins ProSer	Hh	0,0030	rs139382420		
<i>STOX1</i>	47	c.1090C>T	p.Arg364Ter	Hh	0,0001	rs866349614		
	12	c.563C>A	p.Thr188Asn	Hh	0,0037	rs73266500		
	34	c.563C>A	p.Thr188Asn	Hh	0,0037	rs73266500		
	38	c.563C>A	p.Thr188Asn	Hh	0,0037	rs73266500		
	72	c.563C>A	p.Thr188Asn	Hh	0,0037	rs73266500		
<i>STOX2</i>	8	c.63_74del	p.Ala22_Arg25del	Hh	0,0001	rs752333017		
<i>TBX15</i>	7	c.662G>A	p.Arg327His	Hh	0,0005	rs141002143		
	72	c.662G>A	p.Arg327His	Hh	0,0005	rs141002143		
<i>COL18A1</i>	78	c.331G>A	p.Gly111Arg	Hh	0,0017	rs114139997	Extracellular matrix biology	
	98	c.3746G>T	p.Gly1249Val	Hh	0,0000	rs1458664567		
	24	c.4027G>C	p.Gly1343Arg	Hh	0,0014	rs187721798		
	66	c.4027G>C	p.Gly1343Arg	Hh	0,0014	rs187721798		
	86	c.4276_4284delCCCCCT GGG	p.Pro1432_Gly1434del	Hh	0,0017	rs571597296		
<i>COL1A1</i>	40	c.1459C>T	p.Arg487Cys	Hh	ND	ND		
<i>CP</i>	39	c.2687C>T	p.Pro896Leu	Hh	0,0000	rs1415343557		
	88	c.788A>G	p.Asn263Ser	Hh	0,0003	rs150303869		
<i>MMP1</i>	11	c.1389G>A	p.Trp463Ter	Hh	0,0003	rs117105447		
<i>VCAN</i>	10	c.574G>A	p.Gly192Arg	Hh	0,0017	rs141008923		
<i>CCR2</i>	21 m	c.746T>A	p.Met249Lys	Hh	0,0004	rs200491743		Immunological response
<i>CD74</i>	25	c.700G>A	p.Glu234Lys	Hh	0,0007	rs201296907		
<i>IL17A</i>	72	c.311G>A	p.Gly104Glu	Hh	ND	ND		
<i>LGALS13</i>	8	c.221delT	p.Leu74fs	Hh	0,0017	rs371049824		
<i>LILRB1</i>	75	c.1809delG	p.Ala603fs	Hh	0,0002	rs756981570		
<i>LTF</i>	12	c.589delG	p.Glu197fs	Hh	0,0007	rs571988865		
<i>NOD2</i>	2	c.1055A>G	p.His352Arg	Hh	0,0012	rs5743272		
<i>SIGLEC6</i>	84	c.334C>T	p.Arg112Trp	Hh	0,0007	rs78225194		
<i>TNFRSF1 A</i>	75	c.814_850del	p.Ser272_Phe285del	Hh	ND	ND		

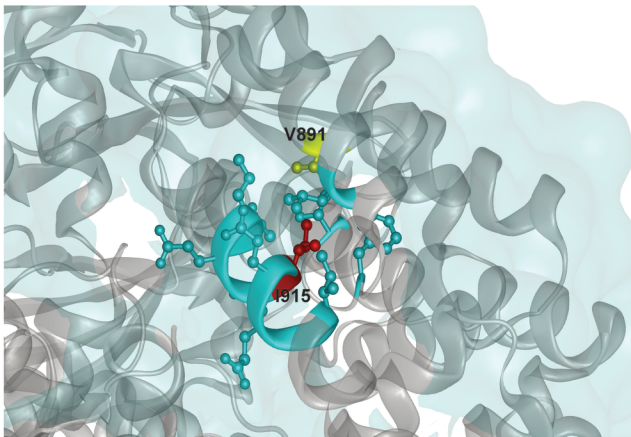
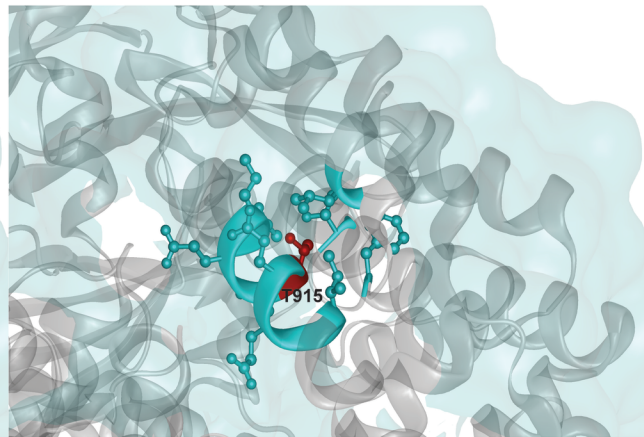
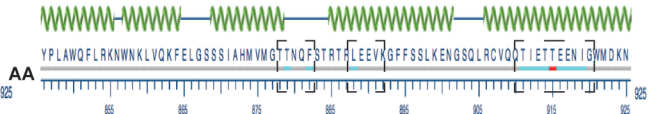
<i>APOB</i>	3	c.11908_11909insCT	p.Trp3970fs	Hh	ND	ND	Lipid metabolism	
	34	c.895T>G	p.Phe299Val	Hh	0,0001	rs72653060		
	57	c.9880T>C	p.Ser3294Pro	Hh	0,0051	rs12720855		
<i>APOC3</i>	47	c.55+1G>A	ND	Hh	0,0013	rs138326449		
<i>CYP1A1</i>	22	c.316delC	p.Arg106fs	Hh	0,0003	rs779460432		
	35	c.316delC	p.Arg106fs	Hh	0,0003	rs779460432		
	5	c.394G>C	p.Ala132Pro	Hh	0,0009	rs35196245		
	79	c.394G>C	p.Ala132Pro	Hh	0,0009	rs35196245		
	11	c.403C>T	p.Arg135Trp	Hh	0,0003	rs45442501		
<i>EPHX1</i>	57	c.454G>A	p.Gly152Ser	Hh	0,0000	rs1364453923		
	52	c.647G>A	p.Gly216Asp	Hh	0,0002	rs542434391		
<i>LEPR</i>	17	c.1835G>A	p.Arg612His	Hh	0,0003	rs144159890		
<i>LIPC</i>	86	c.1064A>G	p.Gln355Arg	Hh	0,0042	rs140272400		
	29	c.1064A>G	p.Gln355Arg	Hh	0,0042	rs140272400		
<i>LIPE</i>	19	c.1210C>T	p.Arg404Cys	Hh	0,0037	rs138983180		
	40	c.2431C>T	p.Arg811Trp	Hh	ND	ND		
<i>PGR</i>	84	c.1498_1500delGCC	p.Ala500del	Hh	ND	ND		
<i>CSAD</i>	93	c.1252C>T	p.Pro418Ser	Hh	0,0001	rs200919847		Others
<i>DNAH11</i>	92	c.12521T>G	p.Leu4174Arg	Hh	0,0009	rs144474011		
	98	c.4060G>T	p.Glu1354Ter	Hh	ND	ND		
	46	c.6302A>G	p.Asn2101Ser	Hh	0,0006	rs201618995		
	57	c.A11059A>G	p.Lys3687Glu	Hh	0,0042	rs72657402		
	72	c.A11059A>G	p.Lys3687Glu	Hh	0,0042	rs72657402		
	82	c.A11059A>G	p.Lys3687Glu	Hh	0,0042	rs72657402		
	93	c.A11059A>G	p.Lys3687Glu	Hh	0,0042	rs72657402		
	96	c.A11059A>G	p.Lys3687Glu	Hh	0,0042	rs72657402		
<i>GPT</i>	45	c.248G>A	p.Arg83His	Hh	0,0002	rs143408057		
<i>MAN1C1</i>	84	c.1670G>T	p.Arg557Leu	HH	0,0000	rs750214180		
<i>PTHLH</i>	16 m	c.71G>A	p.Gly24Glu	Hh	ND	ND		
<i>SOD3</i>	80	c.524G>A	p.Gly175Asp	Hh	0,0012	rs373731075		

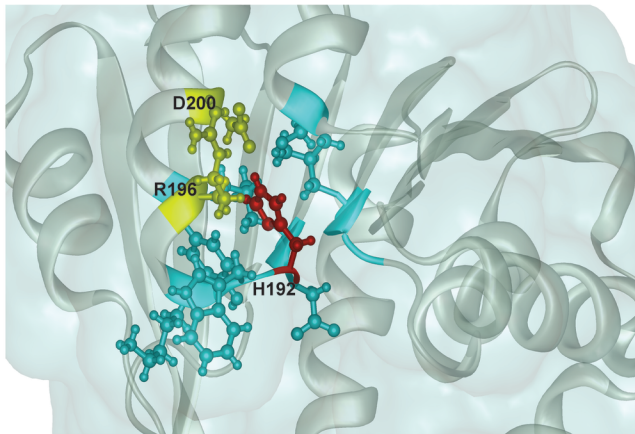
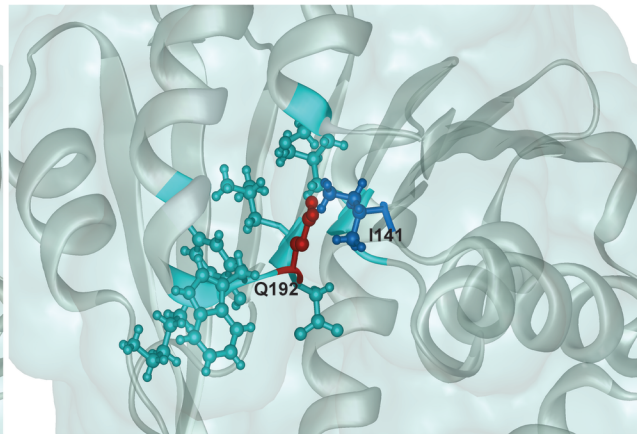
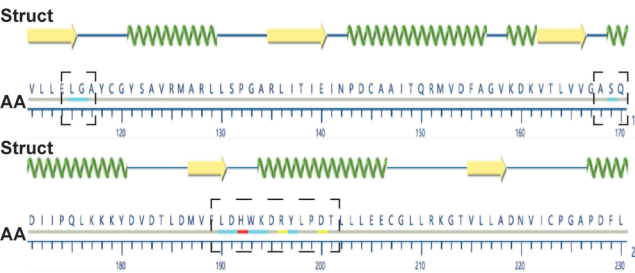
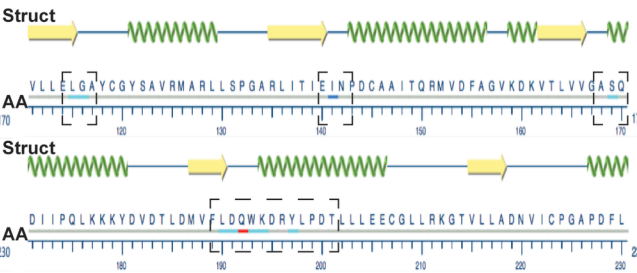
HH: Homozygous. **Hh:** Heterozygous. **ND:** Not described

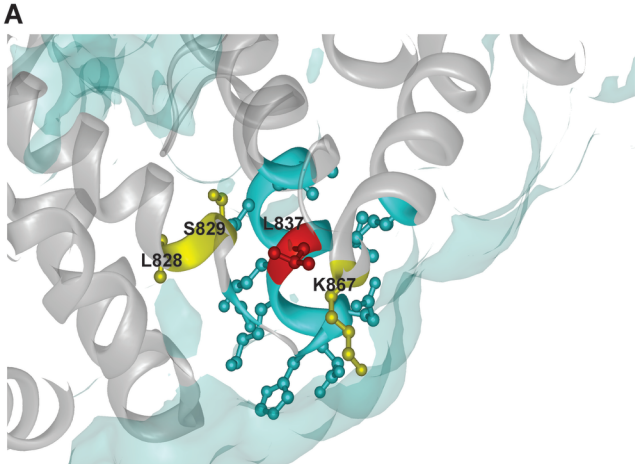
(*): The variant nomenclature refers to that described by Jenny et al. 1987.



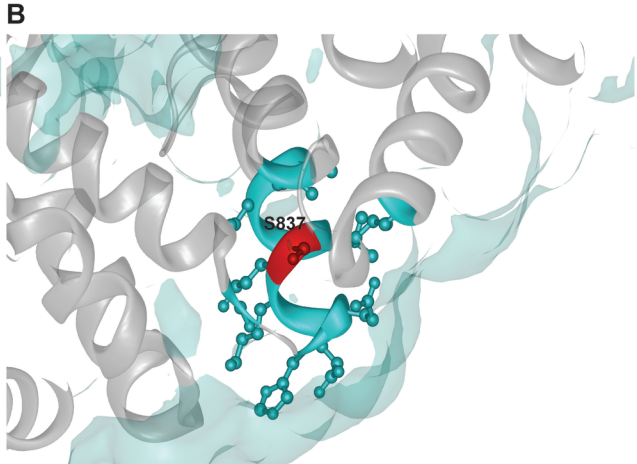
A**B**

A**ERAP1-WT****B****ERAP1-p.Ile915Thr****C****Struct****D****Struct**

A**B****C****D**



ERAP2-WT



ERAP2-p.Leu837Ser

