A founder mutation in P450 reductase from Argentina causes virilization in 46,XY patients.

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Introduction

Cytochrome P450 oxidoreductase (POR) deficiency results in defective steroid production. Mutations in POR cause ambiguous genitalia in both 46,XX and 46,XY patients & adrenal insufficiency with/without bone malformations. A novel homozygous variant c.262G>A/p.G88S in POR was found in four patients in Argentina and no common ancestors have been found between the families. All patients had elevated basal ACTH, 17-OHProg & Prog and normal cortisol with no response to ACTH, and low androstenedione and testosterone. We aimed to perform functional analysis of novel G88S variant in POR in 46,XY patients.

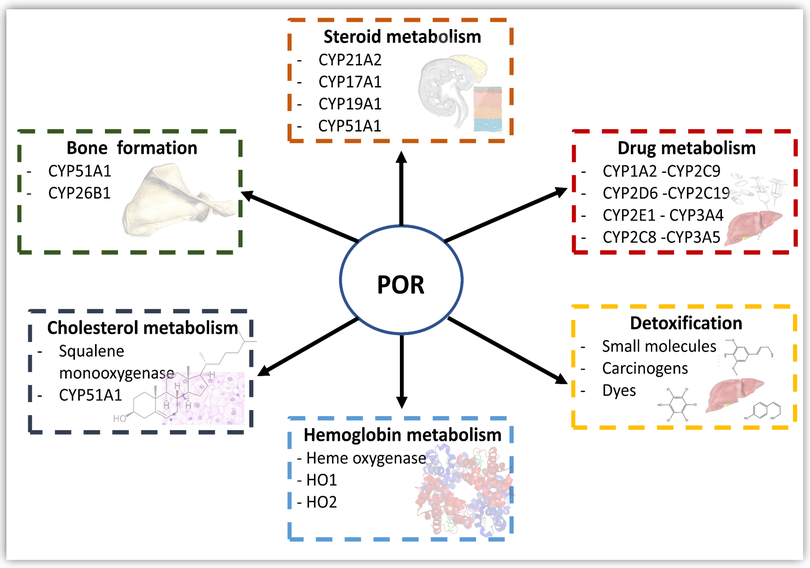


Figure 1. Role of POR in the different metabolic pathways. POR supplies redox equivalents from NADPH to its redox partners via FAD and FMN co-factors. POR is the redox partner for cytochrome P450 proteins located in the endoplasmic reticulum, including the drug metabolizing enzymes, and the enzymes involved in the steroid production.

Cases description: All index cases had a 46,XY karyotype; patients 1-3 were assigned male and patient 4, as female.

Patient 1 had micropenis, perineoscrotal hypospadias, partially fused labioscrotal folds, and palpable inguinal gonads. External Genital Score (EGS) was 4.5 and he had bulbous nose and auricular skin tags.

Patient 2 had micropenis without hypospadias, fused and bifid labioscrotal folds with palpable gonads and EGS of 9. Additionally, he had imperforate anus and colo-vesical fistula.

Patient 3 had micropenis, perineoscrotal hypospadias, partially fused labioscrotal folds with palpable gonads, and EGS of 7. His affected sister (46,XX) presented with primary hypogonadism at pubertal age.

Patient 4 presented with external female genitalia, except for a left inguinal palpable gonad. She had unilateral kidney agenesis and dysmorphic features (bulbous nose, flat nasal bridge, arachnodactyly, thoracic kyphosis).

All patients showed elevated basal ACTH, 17-OH progesterone and progesterone, normal cortisol with no response to ACTH, and low androstenedione and testosterone with no response to hCG. POR deficiency was suspected. None presented radiographic signs of Antley-Bixler syndrome.

Methods

We analyzed the ability of POR wild-type (WT) and G88S to reduce resazurin, MTT, cytochrome c, and activity towards the drug and steroid metabolizing cytochromes P450. POR WT and Gly88Ser were expressed and produced as recombinant proteins in bacteria (E. coli C41(DE3) and combined with recombinant P450 proteins and small molecule substrates for enzyme assays using UV-Vis and fluorescence spectroscopy, analysis of steroids and protein stability studies.

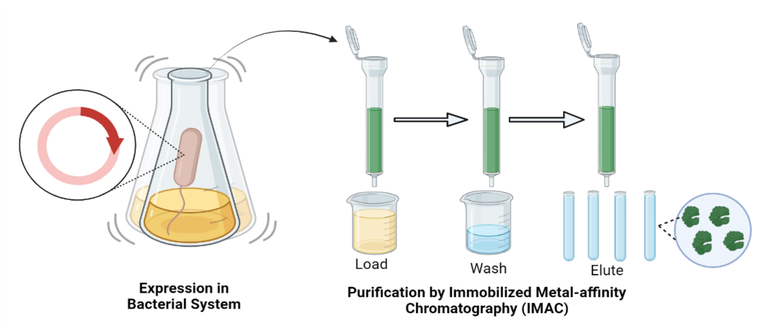
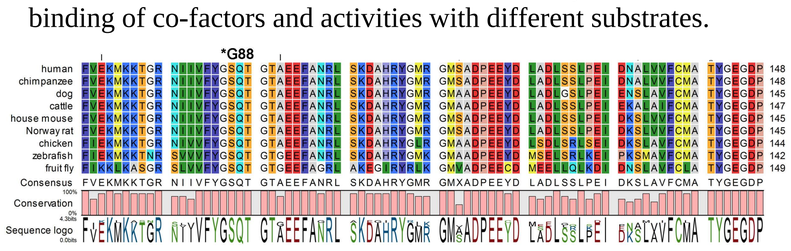


Figure 2. Graphic representation of the workflow.

Results

A novel homozygous variant c.262G>A/p.G88S in POR was found in all four patients and no common ancestors have been found betw een the families. We found severe effects of Gly88Ser mutation on binding of co-factors and activities with different substrates.



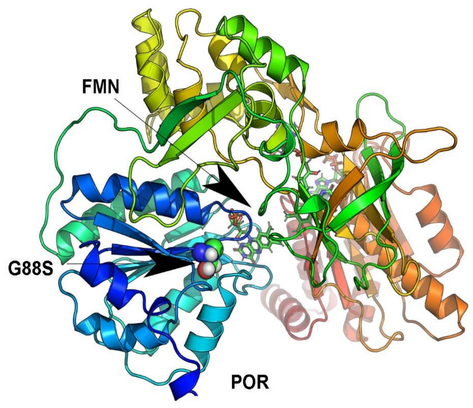


Figure 3. Analysis of POR amino acid conservation. **(A)**. The position G88 is indicated with a star; **(B)** POR structure is illustrated as a ribbon model, the G88S mutation is shown as spheres.

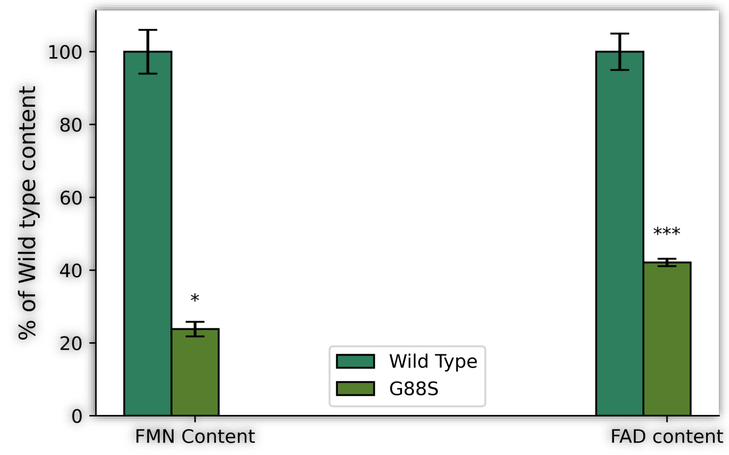
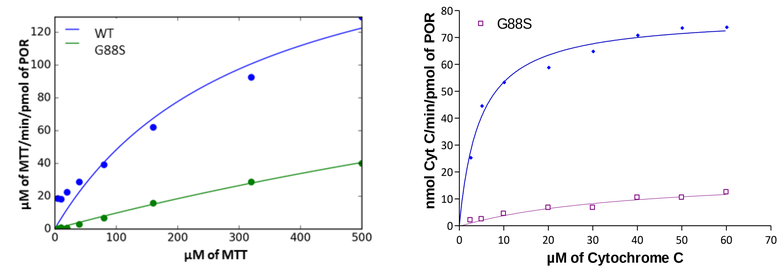


Figure 4. Flavin content of WT and G88S POR. The fluorescence of released FMN/FAD were measured with excitation at 450 nm and emission at 535 nm. The percentages of Flavin content were calculated in comparison to WT set as 100 %. We observed 76% less FAD and 58% less FMN biding in POR G88S.

The G88S mutation in POR severely decreased the efficiency in redu ction of small molecules. The cytochrome c reduction showed a loss of 97% of its activity compared to WT POR. The Rezasurin assay sh owed a decrease of 92%. In the case of the MTT reduction assay, we observed not only a severe loss of activity (a decrease of > 74%) but also a remarkably lower affinity for the substrate.



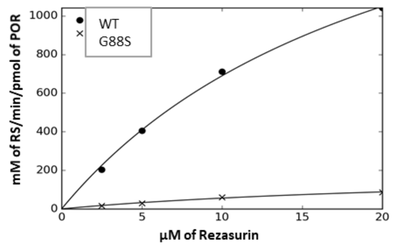


Figure 5. Kinetics of small molecule reduction by WT and P228L POR. (A) Cytochrome c reduction assays. (B) MTT reduction assay. (C) Resaszurin Reduction assay The curves represent the best nonlinear fits to the Michaelis-Menten equation

Table 1. Kinetics parameters for the reactions catalysed by recombinant WT or G88S POR. Vmax/Km was used to compare the activity of WT vs P228L, the WT activity was set at 100%

Parameter	Cytochrome C	MTT	Resazurin
Vmax	20	68	207
KM	40	805	27
Vmax/KM	0.5	0.08	7.6
%	**3**	**26**	**8**

Results

We assessed how the G88S variation in POR affects the activity of four major drug-metabolizing enzymes CYP3A4, CYP3A5, CYP2C9, and CYP2C19.

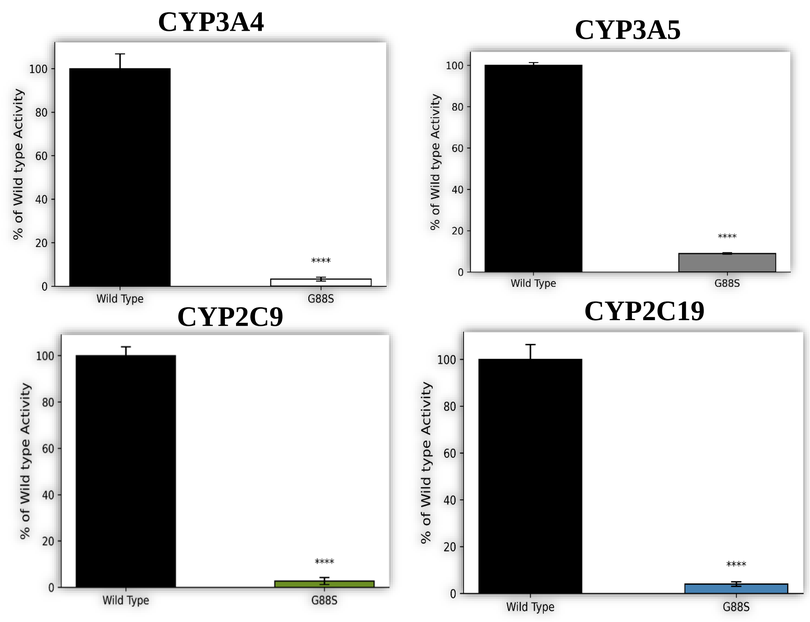


Figure 6: CYP3A4, CYP3A5, CYP2C9, and CYP2C19 activity promoted by WT and G88S POR proteins. Activity with the WT POR was set at hundred percent, and results are given as a percentage of the activity supported by WT POR. All the drug metabolizing CYP450s assays showed that POR variant G88S had more than 90 % inhibition compared to WT activity.

Finally, we assessed how the G88S variation in POR affects the activity of two key enzymes in the steroids production CYP21A2 and CYP17A1

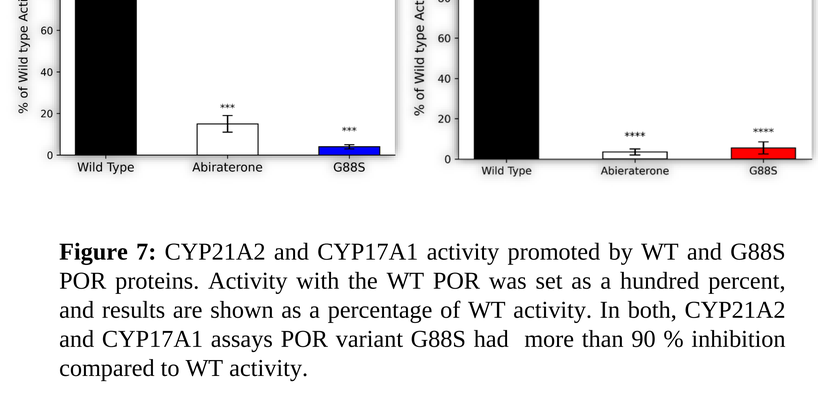


Figure 7: CYP21A2 and CYP17A1 activity promoted by WT and G88S POR proteins. Activity with the WT POR was set as a hundred percent, and results are shown as a percentage of WT activity. In both, CYP21A2 and CYP17A1 assays POR variant G88S had more than 90 % inhibition compared to WT activity.

Discussion and conclusion

The Gly88 residue is located in the FMN binding region of POR, next to a direct contact with FMN. Computational analysis predicted instability in the FMN binding region of POR which was confirmed by lower flavin content in POR-G88S. Therefore, an adverse effect on multiple P450 enzyme activities due to G88S mutation in POR was predicted and then confirmed by experiments with purified recombinant proteins.

The severe impact on the 17,20 lyase activity of CYP17A1and the 21- hydroxylase of CYP21A2 by POR-G88S explains the patient phenotypes and confirms the diagnosis of POR deficiency. The existence of the same variant, not previously described, in 4 unrelated Argentine families raises the possibility of the existence of a founder effect and a diagnostic marker for newborn screening.

Acknowledgements

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