PROFESSOR PHILIPPE J CARON (Orcid ID : 0000-0001-5391-7582)
PROFESSOR FRÉDÉRIQUE SAVAGNER (Orcid ID : 0000-0002-1965-8836)

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NEW VARIANT (Val597Ile) IN TRANSMEMBRANE REGION OF THE TSH RECEPTOR WITH HUMAN CHORIONIC GONADOTROPIN HYPERSENSITIVITY IN FAMILIAL GESTATIONAL HYPERTHYROIDISM.

Short running title: TSHR variant and familial thyrotoxicosis.

[1]Philippe Caron, [1]Stéphanie Broussaud, [2,3]Juan José Galano-Frutos, [2,3,4]Javier Sancho, [5,6]Frédérique Savagner.

Academic degree and contact information:

Philippe Caron, MD, [1]Department of Endocrinology and Metabolic Diseases, Cardiovascular and Metabolic Unit, CHU Larrey, 24 chemin de Pouvourville, TSA 30030, 31059, Toulouse, France. caron.p@chu-toulouse.fr.

Stéphanie Broussaud, MD, [1]Department of Medecine, Hôpital Joseph Ducouing, 15 rue de Varsovie, 31300 Toulouse. SBROUSSAUD@hjd.asso.fr

Juan José Galano-Frutos, PhD, [2,3]Biocomputation and Complex Systems Physics Institute (BIFI)-Joint Units: BIFI-IQFR (CSIC) and GBsC-CSIC, Universidad de Zaragoza, Zaragoza, Spain. Departamento de Bioquímica y

Biología Molecular y Celular, Facultad de Ciencias, Universidad de Zaragoza, Zaragoza, Spain. juanjogf@gmail.com

Javier Sancho, PhD, [2,3,4]Biocomputation and Complex Systems Physics Institute (BIFI)-Joint Units: BIFI-IQFR (CSIC) and GBsC-CSIC, Universidad de Zaragoza, Departamento de Bioquímica y Biología Molecular y Celular, Facultad de Ciencias, Universidad de Zaragoza; Aragon Health Research Institute (IIS Aragón), Universidad de Zaragoza, Zaragoza, Spain. jsancho@unizar.es

Frédérique Savagner, PharmD, PhD, [5,6]Biochemistry and Genetic Laboratory, Federative Institute of Biology, CHU Toulouse, Inserm UMR 1048, Team 6, Institute of Metabolic and Cardiovascular Diseases (I2MC), CHU Rangueil, Toulouse, Institut Cardiomet, Place du Docteur Baylac, 31059 Toulouse, France. savagner.f@chu-toulouse.fr

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Address of corresponding author:
Professor Philippe Caron, M.D.
Department of Endocrinology and Metabolic Diseases, Cardiovascular and Metabolic Unit, CHU Larrey, 24 chemin de Pouvourville, TSA 30030, 31059 Toulouse Cedex, France
Telephone: +33 (0)5 67 77 17 01, Fax: +33 (0)5 67 77 16 72
E-mail: caron.p@chu-toulouse.fr

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SUMMARY

Context: Only two mutations at the Lysine 183 amino acid in the extracellular N-terminal domain of human TSH receptor (hTSHR) have been associated to hypersensitivity to hCG and familial gestational hyperthyroidism.

Patients: A 38-year-old woman was seen during the first trimester of her second pregnancy for thyrotoxicosis with increased fT3 and fT4 concentrations and low TSH levels without anti-TSH receptor antibody. Thyrotoxicosis improved spontaneously during the 2nd trimester and persisted at the 3rd trimester. Similar clinical symptoms (weight loss, nausea, vomiting) were also reported during the first trimester of her first pregnancy and the first pregnancy of her mother.

Results: DNA sequencing of the hTSHR gene of this woman and her mother, identifies a heterozygous variant changing Valine to Isoleucine residue at codon 597 in the transmembrane domain (TMD) of this receptor. *In vitro* functional studies of this variant showed increased constitutive activity in regard to the basal level of cAMP and IP3 production and to the low cell surface expression, while response to TSH was reduced compared to that of the wild type receptor. The Val597Ile variant presented a dose-dependent increase in cAMP response to hGC and human luteinizing hormone (hLH). Simulation of the protein dynamics showed a high structural impact of the Val597Ile variant on helices 3 (TMH3) and 5 (TMH5) of the transmembrane domain participating to constitutive activity and hCG sensitivity.

Conclusion: We describe a new variant in the transmembrane region of the hTSHR gene with increased constitutive activity and hCG hypersensitivity in familial gestational hyperthyroidism.

Key words: gestational thyrotoxicosis, TSHR variant, hCG hypersensitivity, structure-function analysis.

INTRODUCTION

Hyperthyroidism, often times manifesting itself as gestational thyrotoxicosis, occurs in less than 3% of pregnancies (1). The majority of gestational hyperthyroidism is caused by high concentrations of human chorionic gonadotropin which occurs for example during the first trimester of gestation in women with hyperemesis gravidarum. Less common presentations of hyperthyroidism are those due to anti-TSH receptor antibodies in the context of Graves' disease (< 0.5%), due to autonomous thyroid hormone production, or rarely to sporadic mutation of the TSH receptor gene. Moreover familial gestational hyperthyroidism caused by mutations of the hTSHR gene, hypersensitive to hCG, is rare. Only two mutations at the same amino acid residue (Lys183Arg, Lys183Asn) in TSH-binding site of the extracellular N-terminal domain of TSHR have been reported so far (2,3).

The aims of the present article are: 1) to describe a new variant of the TSHR gene with hCG hypersensitivity found in two women of the same family diagnosed with gestational hyperthyroidism, 2) to analyze clinical and hormonal data related to the increased constitutive activity and hypersentivity to hCG and hLH of this mutated receptor in comparison to data observed in previous mutated receptor genes, 3) to explore the structural impact of this variant located on the TMH5 helix of hTSHR by molecular dynamics simulations.

PATIENTS

A 38-year-old woman was seen during the first trimester of her second pregnancy for weight loss (5 kg), nausea and vomiting. Thyroid function test revealed thyrotoxicosis with increased fT3 = 12.7 pmol/L (reference range 3.7 – 6.3 pmol/L) and fT4 = 29.6 pmol/L (10.3 – 16.7 pmol/L) concentrations and low TSH (< 0.05 mU/L) levels without anti-TSH receptor antibody, and normal serum beta hCG level (at day 17 of gestation = 930 IU/L). Thyroid ultrasound showed a normal-sized and homogenous thyroid gland with diffuse hyper-vascularization. The woman did not used multivitamins before or during gestation. She was not treated during the first trimester of gestation, however the thyrotoxicosis improved spontaneously during the 2nd trimester, and persisted during the 3rd trimester of gestation (Figure 1A). The woman gave birth to a girl (3,300 gr; 48 cm), and in the post-partum fT4 and fT3 concentrations returned to their reference ranges and TSH levels were low, in absence of treatment (Table 1). Interestingly, she presented similar

symptoms (nausea and vomiting) with a loss of 6 kg during the first trimester of her first pregnancy.

Her mother had two children and reported similar symptoms during her first pregnancy. At the age of 66 years, she had thyroid function tests in their reference range (fT4 = 13.3 pmol/L, TSH = 0.92 mU/L) and high gonadotropin (LH = 26.8 IU/L, FSH = 85.7 IU/L) levels. The clinical data were collected from information contained in the medical records.

METHODS

Hormonal and genetic analyses

Serum TSH, fT3 and fT4 concentrations were measured with kits routinely used: EIA Abbott Laboratory, Chicago, USA, during pregnancy; and ECLIA, Roche-Diagnostics, France, in post-partum follow-up of the index case and her mother. Beta hCG was measured with kit routinely used (CMIA, Abbott Laboratory, Chicago, USA). After obtaining written informed consent from the index patient and her mother**,** genomic DNA was extracted from peripheral blood leukocytes (Qiagen Blood kit, QIAGEN). Amplification of all the coding sequences of the TSHR gene was performed by PCR using selected primers previously referred (4) as for exon 10 sense: 5'-GCTATGCCAAAGTCAGTATC-3' and reverse: 5'-TAAGTTCCCCTACCATTGTG-3'. Samples were tested by direct Sanger sequencing using BigDye terminator chemistry according to the manufacturer's instructions and analyzed on an ABI 3100 sequencer (all from Applied Biosystems). The whole coding sequence of the hTSHR gene was determined by direct Sanger sequencing.

Functional analyses

FACS analyses were performed on transfected cells incubated for 30 min with 1/400 of a murine anti-hTSHR antibody (2C11, Abcam) followed by incubation with an Alexa Fluor488-labelled goat anti-mouse IgG (Invitrogen). Receptor expression was determined by median fluorescence intensity analysis and reported to set at 100% of the wild-type hTSHR expression (FACscan, Beckton Dickinson).

Transient transfection of COS-7 cells with the hTSHR-pCMV6 plasmid (Origene) containing either the wild-type (WT) or the Val597Ile mutant gene was carried out using 1 µg of plasmid and 0.5 µg of luciferase reporter vector containing cAMP-response element (pGL4.29, Promega) or Gq/11 response element as NF - TA (reference 10959, Addgene). Forty-eight hours after

transfection, cells were incubated in Dulbecco's Modified Eagle Medium +/- bovine TSH (100 mU/mL) for 30 min. Luciferase activity was normalized to β-galactosidase.

The production of cAMP was measured in culture medium after addition of phosphodiesterase inhibitor at baseline and after incubation for 60 minutes with increasing concentrations of bovine TSH, recombinant hCG (C6322) and hLH (L6420), all from Sigma-Aldrich, using the cAMP direct immunoassay kit and manufacturer's recommendations (Ab65355, Abcam). All experiments were performed in duplicate and repeated three times. Cells transfected with empty vector were used as a negative control. Student's t-tests were used for statistical analysis.

Molecular Dynamics simulations

To assess the structural impact of the Val597Ile variant using molecular dynamics (MD) simulations, we developed a homology model of the TSHR transmembrane region using the GPCR-SSFE 2.0 server (5). The model was refined by performing solvated minimization and consecutive short NVT- and NPT-equilibration MD steps (MD-refined model). Its quality was assessed using the MolProbity structure checker (6). Val597Ile mutation was introduced into the refined TSHR-TMD model using the Swiss-Pdb Viewer (7), selecting the best-accommodated rotamer of isoleucine in the mutation position.

Both WT and Val597Ile mutant structures were then subjected to MD simulations with proteins immersed in a lipid bilayer comprising 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC: 122 molecules, 61 in each layer), which was solvated (3689 water molecules) and neutralised (8 chloride ions). Consecutive minimisation (steepest descent, emtol = 1000 kJ/mol), NVT-annealing (500 ps, from 0 to 310 K, single sequence of annealing points) and NPT-equilibration (5 ns, 310 K, 1 atm) steps were performed using the GROMACS 5.1.4 package (8). NPT-productive phases of 2 μs were collected at 37 ºC and 1 atm. The systems were prepared in triplicate both for the wild type and the Val597Ile mutant and a battery of analyses was applied to all the replicas to assess membrane shape and stability along the simulations.

This work conforms to the Declaration of Helsinki Good Clinical Practice guideline. Women have been informed and gave signed informed consent for clinical, hormonal and genetic analysis of their data after explanation of the purpose of the study.

RESULTS

Thyroid function evaluation

The 38-year-old woman had low TSH (0.06 mU/L) with fT4 (19.0 pmol/L) and fT3 (5.5 pmol/L) in the reference range one year before pregnancy. During the first trimester of her second pregnancy, she presented thyrotoxicosis with increased fT4 and fT3 concentrations and low TSH levels (Figure 1A). Thyrotoxicosis improved spontaneously at the end of the first trimester and persisted throughout the second part of the pregnancy. After vaginal delivery and during maternal breast-feeding, thyroid evaluation showed low TSH and fT4 and fT3 levels in the reference range, while urinary iodine levels were low (Table 1). Anti-thyroid antibodies (anti-TSH-receptor and anti-TPO antibodies) were negative before, during and after delivery.

hTSHR mutation and functional analyses

For this woman and her mother, DNA sequencing led to the identification of a heterozygous variant (c.1789 G>A) changing Valine to Isoleucine residue at codon 597 in the exon 10 of the hTSHR gene.

The Val597Ile variant in the transmembrane region of hTSHR displayed a reduced expression level on the cell surface (28% of the wild-type receptor, Figure 1B). This mutated receptor showed increased constitutive activity with regard to the basal level of cAMP and IP3 production (2 to 2.5-fold higher), while the response to TSH was reduced compared to that of the wild-type receptor (50% on average), and related to its low cell-surface expression.

This Val597Ile variant presented a dose-dependent increase in cAMP production in response to hCG and hLH whereas the wild-type receptor was insensitive to those hormones except at high concentrations of hCG (Figure 1C).

Structural impact of the Val597Ile variant on the TSH receptor-transmembrane domain

The transmembrane domain of the TSH receptor consist of seven α-helices that are packed with each other. The structural impact of the Val597Ile substitution on the fifth transmembrane helix (TMH5) can be assessed by monitoring the integrity of this helix and that of its packing with neighbouring helices 3 and 6 (TMH3 and TMH6) along the simulations of both the wild type and the mutant receptor structure. TMH5 integrity has been evaluated from the dihedral angles of the residues in the helix, as depicted in a Ramachandran plot, and by comparing the number of

residues that are part of the helix at the beginning of the simulation with that at the end. In addition, the retention or lost of the native helix/helix packing in the mutant receptor versus that one shown by the wild type protein can be inferred comparing the distances between key residues located in adjacent helices of either structure.

At the beginning of the MD simulations, helix TMH5 was a regular helix with all dihedral angles lying in the allowed region of the Ramachandran plot (Figure 2A, panel A1). The structural effect of the Val597Ile mutation was a clear distortion of the helix and of its interaction with neighboring helices 3 (TMH3) and 6 (TMH6), which could be observed in the three replicas of the Val597Ile mutant (Figure 2A, panel A3) but not in the WT replicas (Figure 2A, panel A2). The occurrence of residues outside the initial helical region in the three replicas of the WT-TMD along the simulation varied from 0.2 to 0.7 % (mean of 0.4 %; see bars in Figure 2A, panel A2). In contrast, for the mutant receptor, the residues outside the initial helical position in the three replicas amounted to 0.8-5.3 % (mean of 3.3 %; see bars in Figure 2A, panel A3). This was reflected along the mutant trajectories in the large fluctuation in the alpha-helical content of residues in TMH5 (residues 580 to 605), compared to the small fluctuations observed in the WT trajectories.

To monitor the distortion of inter-helical interactions, the distances between key residues at the N-terminus, central position and C-terminus of helix TMH5 (L580, A593, Y605, respectively) with facing residues of packing helices TMH3 and TMH6 were determined (Figure 2B). The Val597Ile mutation distorted the packing of TMH5 with helices TMH3 and TMH6, increased the distances between TMH5 and those helices and, probably as a consequence, became less stable, some of its residues adopting non-helical angles along the simulation.

DISCUSSION

We describe a new variant (Val597Ile) in the transmembrane region of the TSH receptor gene with hCG hypersensitivity and increased constitutive activity in two women with familial gestational thyrotoxicosis. To the best of our knowledge, only two mutations at codon 183 of the ligand-binding site in the extra-cellular N-terminal domain of the hTSHR gene have been reported in the literature, in women with early, prolonged and/or severe gestational thyrotoxicosis (2,3). Functional characterization of those Lys183Arg and Lys183Asn mutations revealed

increased sensitivity to hCG responsible for gestational thyrotoxicosis. In this article we describe the Val597Ile variant located in the transmembrane helix TMH5 of the receptor, which is a critical region for membrane targeting and signal transduction, but not for ligand binding to hTSHR.

Basal and TSH-stimulated production of cAMP in cells expressing the Lys183Arg mutated receptor was similar to that of cells expressing the wild-type receptor (2). The absence of any constitutive activity of the Lys183Arg receptor explains that thyroid function tests are normal when analyzed or performed between pregnancies in those women. *In vitro* studies of the Val597Ile mutated receptor reveal increased intrinsic activity which can explain the low TSH and free thyroid hormone concentrations in the reference range present in the index case before pregnancy and during long-term post-partum (Table 1). Functional studies of the Val 597Ile receptor are similar to those of the Val597Phe mutant, attesting a marked increase in ligand-independent receptor activity and associated with a dominant non autoimmune hyperthyroidism (9). Another mutation (Val597Leu) at the same codon was also associated with severe thyrotoxicosis in one infant (10). The Val597Ile mutation showed a significant increase in receptor constitutive activity but significantly decreased receptor expression at the membrane that led to a diminished response to TSH. Therefore, the difference in specific constitutive activity between Lys183Asp and Val597Ile can account for the difference in hormonal phenotype during the non-pregnant state of the reported woman. Finally, as already shown for the wild type TSH receptor, we can make the hypothesis that the new variant (Val597Ile) of the TSH receptor is differently stimulated by certain hCG isoforme such as asialo-, nicked and hyperglycosylated hCG molecules, with levels variable during the course of gestation. Despite the fact that high concentrations of hLH activate the Val597Ile receptor *in vitro*, the serum concentration of LH after the menopause is probably too low to cause significant activation of the Val597Ile mutated receptor. Indeed, the mother of our index case had normal TSH concentrations after menopause despite high gonadotropin levels.

As related to structure-function analysis (11,12), activating mutations located in the extracellular loop of G-protein coupled receptors need the extracellular domain to achieve constitutive activity whereas activating mutations in transmembrane helix and intracellular loops show high-level constitutive activity independently of the presence of the extracellular domain of the receptor. Several mutations in the transmembrane region of the FSH receptor causing constitutive activity in women with spontaneous ovarian hyperstimulation syndrome have been shown to render the mutant sensitive to hCG (13). In a recent review (14), a structural model of the entire human TSH

receptor was proposed, the TMD of which closely resembles our present model. A key role is attributed to interactions between the regular helix TMH5 and helices TMH3 and TMH6 in regulating the signaling activity of the receptor. More specifically, contacts between residues A593 (TMH5), V509 (TMH3) and M637 (TMH6) appear to be essential for triggering the active state in hTSHR, and constitutively activating mutations affecting those residues have been described (15, 16). Our simulations of the wild type and Val597Ile mutant of the hTSHR-TMD clearly show that this mutation decreases the stability of TMH5 and modifies its packing onto helices TMH6 and TMH3, and the distance between the key central residues of helices TMH5 and TMH3. The increased distance between TMH3 and TMH5, especially for amino acid residues close to the extracellular space, could also be proposed to change substrate affinity and oligomerization efficiency, as previously suggested for TMH5 (16). This structural change can participate to constitutive activity and increased hCG sensitivity of the Val597Ile variant of the hTSHR gene in familial gestational hyperthyroidism.

Finally, in the reported family the genotype alone could not explain the variable phenotype presentation of patients affected by Val597Ile mutation in the non-pregnant state. According to epidemiological studies, France is no longer considered a country at risk of iodine deficiency (18–20). However urinary iodine excretion of the index case was low during long-term breastfeeding (27.2 and 12.3 µg/L). In addition to constitutive activation and hCG hypersensitivity of the cAMP pathway secondary to Val597Ile mutation of hTSHR and TMH5 distortion, the variable low iodine intake could influence thyroid dysfunction aside from pregnancy and probably the severity of gestational thyrotoxicosis.

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Table 1: Thyroid function parameters and urinary iodine concentrations in the post-partum during long-term breastfeeding of the index case.

After birth	42 days	185 days	12 months	19 months	Reference range*
Free T4	23.7	19.6	18.1	20.7	12.0 – 21.9 pmol/L
Free T3	6.9	5.1	5.1	5.7	3.1 – 6.6 pmol/L
TSH	< 0.05	0.06	0.03	0.02	0.2- 4.2 mU/L
Urinary iodine concentration			27.2	12.3	100 –199 µg/L

* Cobas 8000 ECLIA

Figures legends

Figure 1: Thyroid function tests of the proband and *in vitro* functional studies of the Wild Type and Val597Ile mutant Thyroid Stimulating Hormone Receptor.

1A: serum fT3 and fT4 concentrations during pregnancy (weeks of gestation). Green and grey boxes are reference values for fT3 and fT4 concentrations from EIA Abbott Laboratory, Chicago, USA., respectively.

1B: Functional analyses of hTSHR mutant after TSH, hCG or hLH stimulation. COS-7 cells were transiently transfected with empty vector (red dot representative of all stimulated mock), wild type (blue dots) and mutant TSHR (grey dots). Cells were stimulated by increasing concentration of bovine TSH, hCG or LH and extracellular cAMP was determined. Each curve represents 3 independent experiments in duplicate and data are means +/- SEM. **1C:** Activation of adenylate cyclase and Gq/11 48h after wild-type- and Val597Ile-hTSHR transfection. Empty vectors are represented as mock. Basal activity of wild-type was set at 100%. Cells were stimulated with bovine TSH. Data represents the mean ± SEM (n=6); * $p < 0.05$ and ** $p < 0.01$.

Figure 2: Molecular dynamics simulation of the mutant Thyroid Stimulating Hormone Receptor

A: Ramachandran plots for TMH5 residues along the simulations. (A1) Scatter plots of the Phi/Psi angles of TMH5, TMH3 and TMH6 in the refined TSHR-TMD homology model, showing an ellipse encompassing all residues. The ellipse is taken here as a representation of the allowed helical region, and used to compare the Ramachandran plots of the simulations. (A2,A3) Ramachandran plots for TMH5 (residues 580-605) in the WT and Val597Ile (V597I) simulations (three replicas: r1, r2 and r3), respectively. The plots display the ellipse obtained as described in (A). Insets display bars with the statistics of Phi/Psi points falling either inside or outside the allowed helical region (ellipse). **B:** Inter-helical distances. Distance averages (± SD) along the simulation time between the mutation carrier helix (TMH5: red) and their closest neighboring helices TMH3 (pale blue) and TMH6 (blue) (TMH5-TMH3, TMH5-TMH6) at three different relative positions. (B1) top (close to the extracellular space): distances between alpha carbon in N-ter-TMH5 residue (L580) versus alpha carbons in N-ter-TMH3 (C494) and C-ter-TMH6 (L649)); (B2) center (distances between alpha carbon in A593 (TMH5) versus alpha carbons in V509

(TMH3) and M637 (TMH6)); and (B3) bottom (close to the cytoplasm): distance between alpha carbon in C-ter-TMH5 (Y605) versus alpha carbons in C-ter-TMH3 (W520) and N-ter-TMH6 (A623). The indicated positions are shown in the cartoons. The same distances but for the TSHR-TMD model have been also calculated and shown for comparison purposes (green bars).

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Author contributions

PhC and SB were responsible for collecting clinical data from the women; PhC wrote the manuscript; JJ G-F and J S were responsible to collect and to present molecular dynamics simulations, reviewed and approved the final version of the manuscript, SG reviewed and approved the manuscript. FS was responsible for collecting DNA data from the family, and wrote the manuscript.

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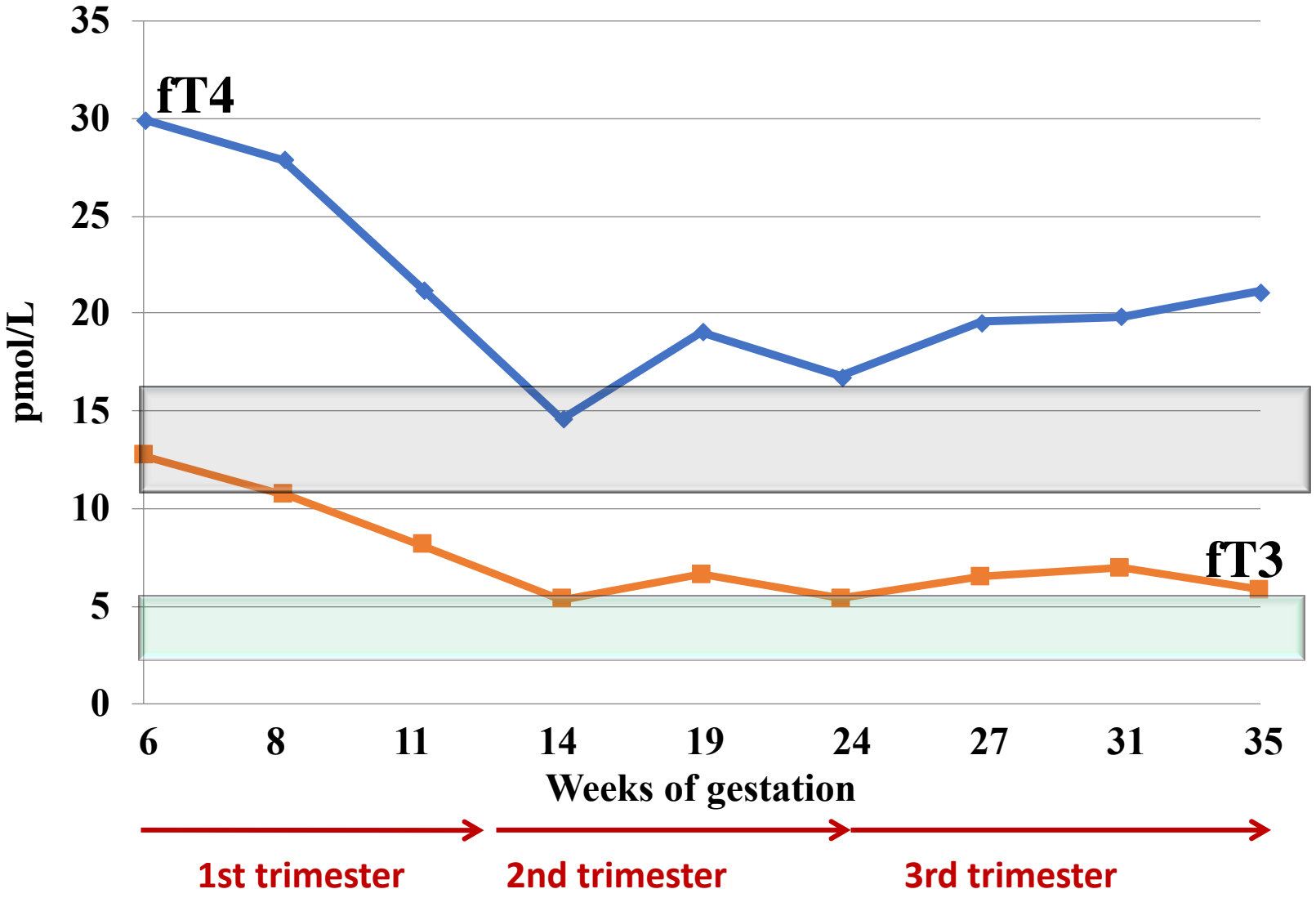

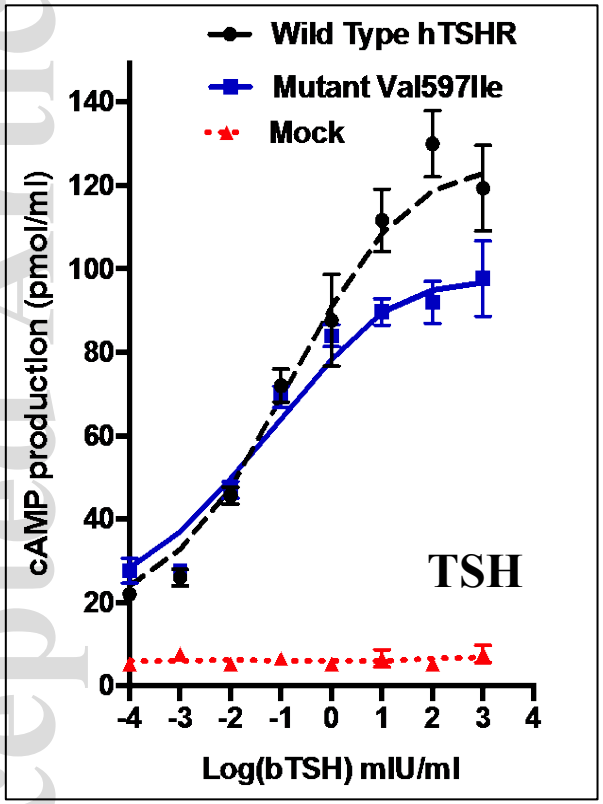

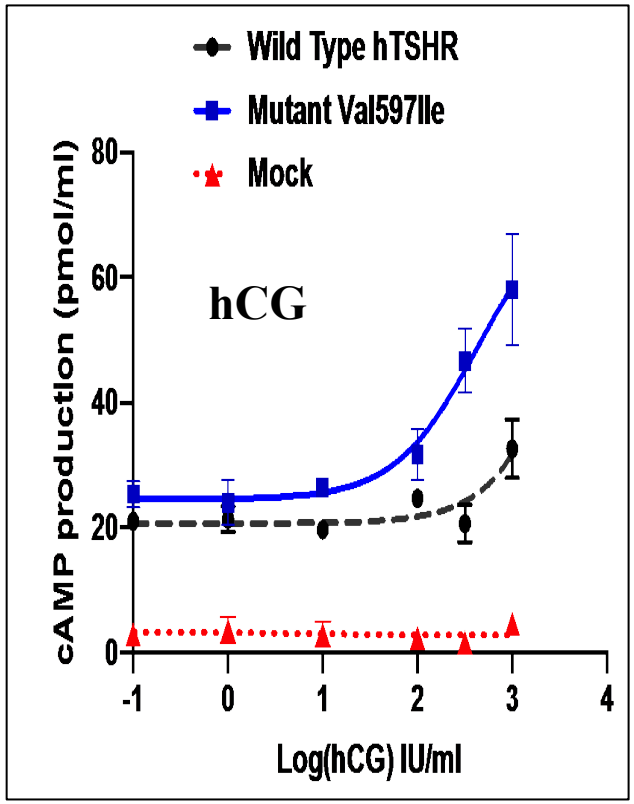

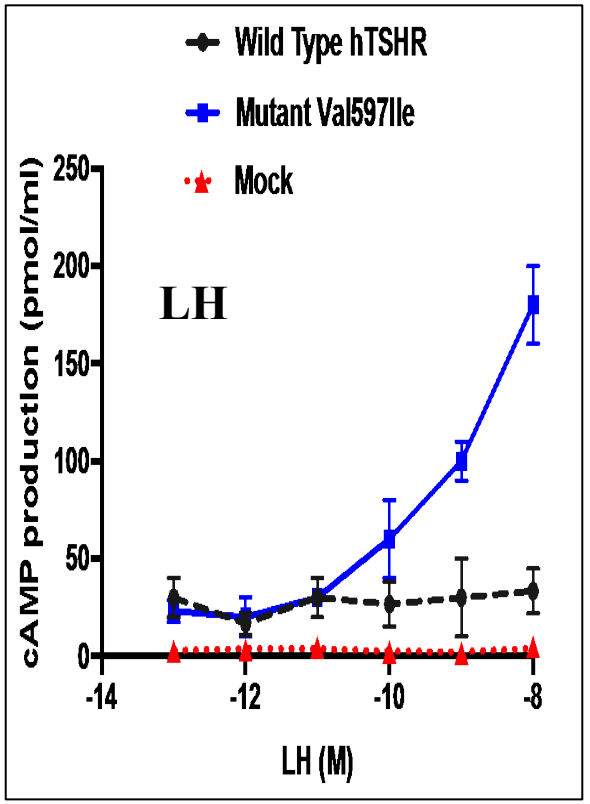

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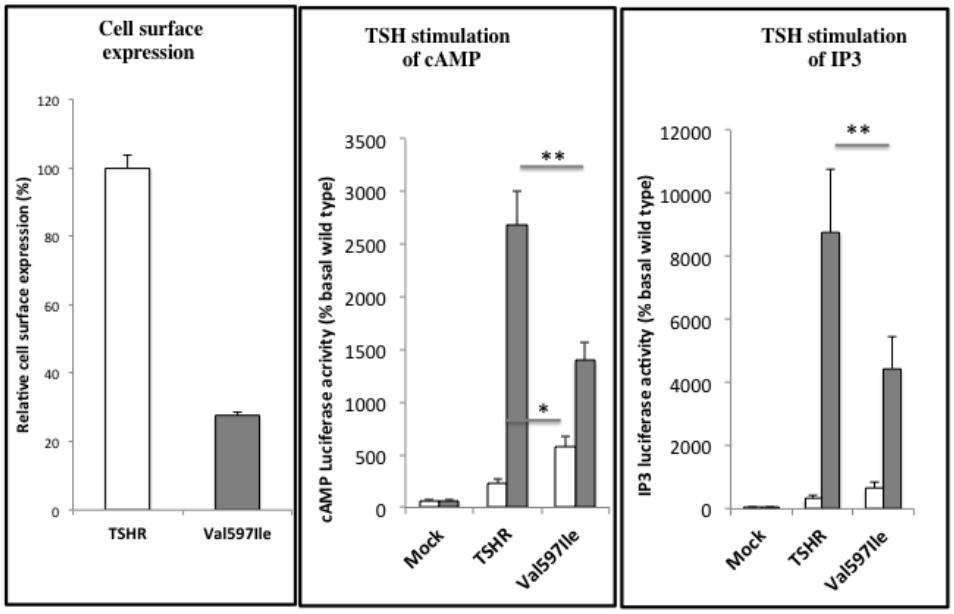

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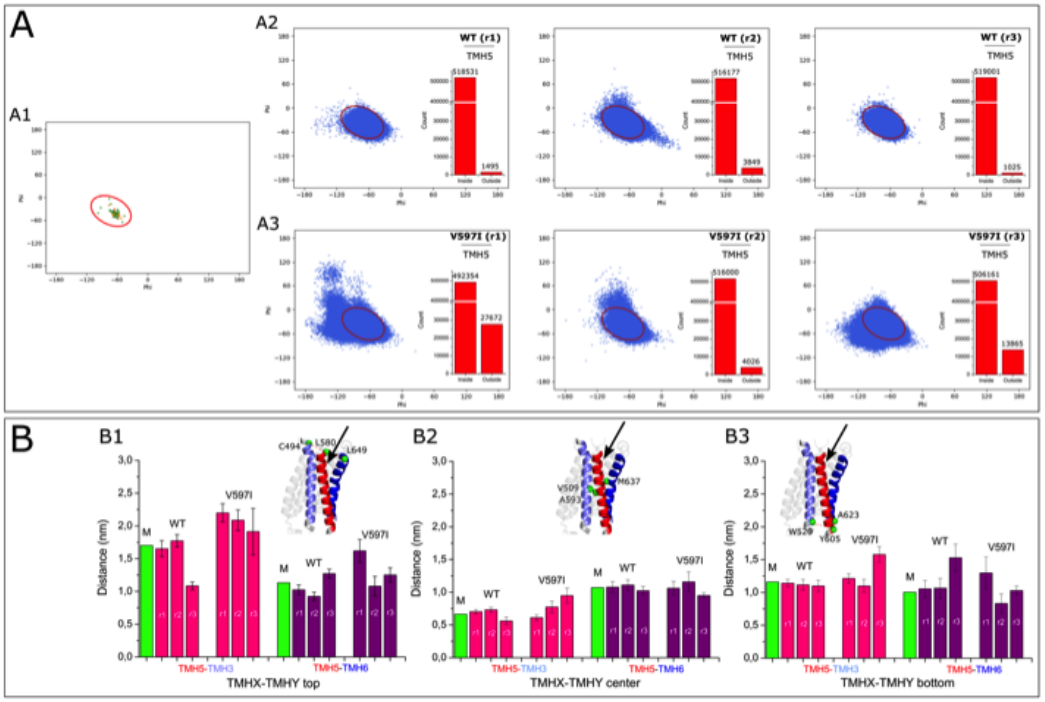

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