**RESEARCH ARTICLE**

**GGCX variants leading to biallelic deficiency to γ-carboxylate GRP cause skin laxity in VKCFD1 patients**

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**Abstract**

γ-Glutamyl carboxylase (GGCX) catalyzes the γ-carboxylation of 15 different vitamin K dependent (VKD) proteins. Pathogenic variants in GGCX cause a rare hereditary bleeding disorder called Vitamin K dependent coagulation factor deficiency type 1 (VKCFD1). In addition to bleedings, some VKCFD1 patients develop skin laxity and skeletal dysmorphologies. However, the pathophysiological mechanisms underlying these non-hemorrhagic phenotypes remain elusive. Therefore, we have analyzed 20 pathogenic GGCX variants on their ability to γ-carboxylate six non-hemostatic VKD proteins in an in vitro assay, where GGCX variants were expressed in GGCX−/− cells and levels of γ-carboxylated co-expressed VKD proteins were detected by a functional ELISA. We observed that GGCX variants causing markedly reduced γ-carboxylation of Gla rich protein (GRP) in vitro were reported in patients with skin laxity. Reduced levels of γ-carboxylated Matrix gla protein (MGP) are not exclusive for causing skeletal dysmorphologies in VKCFD1 patients. In silico docking of vitamin K hydroquinone on a GGCX model revealed a binding site, which was validated by in vitro assays. GGCX variants affecting this site result in disability to γ-carboxylate VKD proteins and hence are involved in the most severe phenotypes. This genotype-phenotype analysis will help to understand the development of non-hemorrhagic phenotypes and hence improve treatment in VKCFD1 patients.

**KEYWORDS**

GGCX, MGP, PXE-like, UCMA/GRP, VKCFD1

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**1 | INTRODUCTION**

Vitamin K-dependent coagulation factor deficiency type 1 (VKCFD1; MIM# 277450) is a rare hereditary bleeding disorder caused by pathogenic variants in the γ-Glutamyl carboxylase (GGCX) gene (Watzka et al., 2014). GGCX is an enzyme located in the ER that converts specific glutamic acid residues (Glu) into γ-carboxyglutamic acid residues (Gla) in vitamin K dependent (VKD) proteins (Rishavy & Berkner, 2012; Vermeer, 1990). This posttranslational modification is essential for the proper functioning of VKD proteins. Seven out of 15 VKD proteins have functions in the coagulation cascade including coagulation factor II (FII), VII (FVII), IX (FIX), X (FX), Proteins C (PC), S, and Z (Kurachi & Davie,
Therefore, VKCFD1 patients harboring pathogenic GGCX variants in homozygous or compound heterozygous states are mainly diagnosed with bleeding symptoms. This hemorrhagic phenotype is caused by the impaired ability of GGCX variants to \(\gamma\)-carboxylate VKD clotting factors thereby leading to their decreased activity. To prevent and treat bleeding symptoms, VKCFD1 patients are treated with life-long administration of vitamin K, which improves activities of VKD clotting factors in most cases. In addition to the hemorrhagic phenotype (Brenner et al., 1998; Darghouth et al., 2006; Spronk et al., 2000) some VKCFD1 patients were reported with additional non-hemorrhagic phenotypes such as Pseudoxanthoma elasticum (PXE)-like syndrome, where patients develop skin hyper-laxity and folds characterized by severe fragmentation and calcification of elastic fibers. Additionally, cardiac abnormalities as congenital atrial septal defects, calcified peripheral arteries, and/or subclinical atherosclerosis were diagnosed (Li, Grange, et al., 2009; Vanakker et al., 2007; Watzka et al., 2014). Moreover, skeletal defects were described in some patients such as midfacial hypoplasia or Keutel-like syndrome, which is characterized by brachytelephalangy of the fingers and facial dysmorphologies (Vilder et al., 2017). These additional phenotypes are most likely developed due to the under-carboxylation of one or a combination of several non-hemostatic VKD proteins. Non-hemostatic VKD proteins include matrix Gla protein (MGP), upper zone of the growth plate and cartilage matrix-associated protein (UCMA/GRP), osteocalcin (BGLAP), prolinc-rich Gla proteins (PRGPs) 1 and 2, and transmembrane Gla proteins (TMGs) 3 and 4, and growth arrest-specific 6 (GAS6) (Kulman et al., 2001, 2007; Manirolelli et al., 1993; Poser et al., 1980; Price et al., 1987; Viegas et al., 2009, 2015). These proteins have diverse functions where BGLAP, MGP, and GRP have a role in the regulation of physiological calcification in bone or soft tissues (Lacombe & Ferron, 2015; Willems et al., 2014). GAS6 is a ligand of the TAM receptor (Tyro, Axl, and Mer) and has functions in cell signaling as for example in proliferation and platelet aggregation (Berker & Runge, 2004). The function of the four VKD transmembrane proteins PRGP1 and 2, TMG3 and 4 is not yet clear.

The mechanism of how some specific GGCX variants lead to the development of non-hemorrhagic phenotypes is unknown. Moreover, it is unclear whether life-long treatment with vitamin K is sufficient to rescue or to prevent non-hemorrhagic phenotypes. Hence, the identification of the under-carboxylated VKD protein/s associated with the corresponding GGCX genotype and their responsiveness to vitamin K administration will be a crucial step to understanding the mechanism of developing non-hemorrhagic phenotype/s.

In the present study, we have evaluated the effect of 20 reported GGCX variants on their ability to \(\gamma\)-carboxylate six different non-hemostatic VKD proteins in the presence of increasing vitamin K concentrations in a GGCX\(^{-/-}\) HEK293T cell line. Furthermore, the \(\gamma\)-carboxylation status of each GGCX variant obtained from our in vitro data was correlated with the previously reported non-hemorrhagic phenotype diagnosed in the VKCFD1 patient. Most notably, we identified that GGCX variants with markedly reduced ability to \(\gamma\)-carboxylate GRP are reported in VKCFD1 patients, who developed the PXE-like phenotype. Additionally, we performed a KH2 docking on a GGCX in silico model, on which we have identified the KH2 binding site. Pathogenic GGCX variants affecting this site showed either loss-of-function or extremely reduced ability to \(\gamma\)-carboxylate VKD proteins, which cannot be rescued by vitamin K administration. Altogether, the knowledge of causative VKD proteins and the responsiveness of GGCX variants to vitamin K will help to optimize the treatment of VKCFD1 patients in the future.

## METHODS

### 2.1 Construction of bicistronic human GGCX/VKD protein expression vectors

To express the cDNAs from a VKD protein together with a GGCX variant from one transcript, the bicistronic pIRES vector (Clontech) was chosen for our expression study. The cDNAs of VKD proteins (UCMA/GRP, MGP, BGLAP, PRGP1, TMG4, and GAS6) together with a C-terminal c-myc tag were cloned into the multiple cloning site (MCS) A of the pIRES vector. The wild-type (wt) human GGCX (hGGCX) cDNA was cloned into the second MCS of the bicistronic vector (MSC B). GGCX variants were introduced into the GGCX wt sequence by site-directed mutagenesis PCR using PFU turbo DNA polymerase according to the manufacturer’s instructions (Agilent technologies, #600250). Table S1 contains the primer pairs used for mutagenesis PCR. The reference number of GGCX DNA (LRG_592) and protein sequences were RefSeq NM_000821.7 and NP_000821.2, respectively. All GGCX variants were validated by VariantValidator online tool (https://variantvalidator.org/; Accessed April 9, 2021).

### 2.2 Measurement of \(\gamma\)-carboxylation of non-hemostatic VKD proteins

CRISPR/CAS9 gene-editing technology was used to generate a GGCX\(^{-/-}\) HEK293T cell line to eliminate endogenous GGCX activity as previously described (Ghosh et al., 2021). In this GGCX\(^{-/-}\) HEK293T cell line, pIRES vectors harboring cDNAs of GGCX wt or variants were transfected together with one myc-tagged VKD protein. Four hours post-transfection cells were treated with different concentrations of vitamin K1 (K1; 0.1, 0.316, 1.3, 3.62, 10, 31.6, and 100 \(\mu\)M; Sigma-Aldrich, #V3501). Cell media and lysates were collected 48 h after transfection to measure \(\gamma\)-carboxylation of VKD proteins by a sandwich ELISA. Cell media were used to measure \(\gamma\)-carboxylation of GRP, MGP, BGLAP, and GAS6, which are secretory VKD proteins. Cell lysates were used to measure \(\gamma\)-carboxylation of PRGP1 and TMG4 because they are transmembrane proteins. Media and lysates of untransfected cells served as negative controls. For the sandwich ELISA, 96-well plates were coated with an anti-myc antibody (Applied Biological Materials, G077) and were incubated overnight at 4°C. The day after, wells were washed 3× with wash buffer (1× PBS, pH 7.4, 3 mM MgCl2, 0.05% Tween 20) and blocked in 2% BSA for 2 h. Samples (media or lysates) were diluted in sample dilution buffer...
(1× PBS, pH 7.4, 0.1% BSA) and incubated overnight. This was followed by 3× washing and 3 h incubation with an anti-Gla antibody, which is directed against Gla residues within the Gla domain (Sekisui, 3570). For MGP we used an MGP-specific Gla antibody (MaGla G8A.1 lot3, gift from Prof. Leon Schurgers), which recognizes the γ-carboxylated fold of MGP. After antibody incubation, wells were washed 3× and incubated with HRP-conjugated antibody (Dako, P0260) for 1 h at RT followed by 3× washing and incubation with an HRP-dependent chemiluminescence substrate (Roche, 11582950001) followed by luminescence measurement by a plate reader (Synergy 2, BioTek).

2.3 | Immunofluorescence staining

GGCX<sup>−/−</sup> cells were seeded on gelatin-coated cover-slips and transfected with vectors containing GGCX wt or variant cDNA. Twenty-four hours after transfection, cells were washed with PBS, fixed with 4% paraformaldehyde for 10 min and then blocked for 30 min with 10% FBS in 1× PBS. Cells were incubated overnight with primary antibodies against GGCX (Abcam, ab170921) and against Protein disulfide isomerase (PDI, Thermo Fisher Scientific, MA3-019). Secondary Alexa Fluor 488 or 594-conjugated antibodies were incubated for 1 h at RT in the dark. Mounting was performed with ProLong Glass (Thermo Fisher Scientific) including Hoechst33342. Images were taken by Apotome2 (Zeiss) and Zen software was used to calculate global Pearson’s correlation coefficient (PCC).

2.4 | Generation of the GGCX structural model and in silico docking of vitamin K

GGCX is a multi-pass membrane protein with 758 amino acid residues. Due to the absence of biophysical structure or adequate homologous structural templates, an hGGCX model was generated on the I-TASSER threading modeling server (https://zhanglab.ccmb.med.umich.edu/I-TASSER/; Roy et al., 2010) by submitting the GGCX protein sequence (NP_000812.2) under default conditions. The model1 with the best C-score was used for further analysis but its N- and C-termini had the same orientation. This contrasts with previous in vitro findings of J. Tie et al. (2000), who showed an opposite location of the N and C-termini. Their analysis revealed that the N-terminus is located in the cytoplasm, whereas the C-terminus is in the ER lumen where γ-carboxylation is taking place. So far this is the only experimental evidence of the orientation of the termini for the GGCX protein. Therefore, the N-terminal residues 1–120 were re-modeled separately in I-TASSER and manually replaced onto model 1 to obtain the orientation of N and C-termini according to the previously published in vitro data.

A membrane-embedded simulation was performed on the final re-modeled structure in a phosphatidylcholine bilayer to equilibrate the model using md_memsim macro embedded in YASARA. The equilibrated model was then docked with K<sub>1</sub> hydroquinone (PubChem ID: 5280585) using the AutoDock module embedded as a macro in YASARA. The top docking poses were analyzed and one docking pose was selected as the final complex based on rational elimination as discussed in Figure S4. The selected docked complex was further subjected to membrane-embedded simulation for a total of 250 ns including equilibration and production phase (>100 ns). The selected variants GGCX:p.(Ser300Phe) and GGCX:p.(Met174Arg) were introduced into the equilibrated docked complex model and both mutated complexes were simulated individually for >100 ns to understand the structural effect of the variants on GGCX. All simulations, visual rendering, and structural analysis were performed on the YASARA version 18.2.24 platform (Land & Humble, 2018; Figure S1). All structural bioinformatics prediction methods used in this study have been conducted on previously cited and validated software’s on open access web servers and conform to the journal guidelines.

3 | RESULTS

3.1 | Evaluation of γ-carboxylation of non-hemostatic VKD proteins

We have analyzed the effect of 20 previously reported GGCX variants on their ability to γ-carboxylate six different non-hemostatic VKD proteins. The co-expression of GGCX variants and VKD proteins was performed in a GGCX<sup>−/−</sup> HEK293T cell line, which is lacking endogenous GGCX activity. GRP, MGP, and BGLAP were included in our study because these proteins are known to function as calcification regulators. PRG1 and TMG4 were included because their function is unknown. Furthermore, GAS6 was included as it has functions in cell signaling through TAM receptors, which is associated with cardiovascular pathology. In the following sections, GGCX variants and their ability to γ-carboxylate VKD proteins measured by in vitro assay were correlated with the previously reported patient’s phenotypes, which include skin hyperlaxity, facial dysmorphologies, and cardiac abnormalities.

3.2 | The skin phenotype is associated with the biallelic deficiency of GGCX to γ-carboxylate GRP

Four VKCFD1 patients with a complete GGCX genotype were reported with a severe PXE-like phenotype including skin hyper-laxity and skin folds. The age of onset varied from 16 to 48 years (Goldsmith et al., 1982; Li, Grange, et al., 2009; Li, Schurgers, et al., 2009; Vanacker et al., 2007). These four patients were found to be compound heterozygous for pathogenic GGCX variants, where one allele has a loss-of-function or a nonsense variant in GGCX. This results in monoallelic loss to γ-carboxylate VKD proteins. The other alleles harbor one of the GGCX variants GGCX:p.(Arg83Trp), GGCX:p.(Val255Met), GGCX:p.(Gly537Ala), or GGCX:p.(Gly558Arg) that exhibited levels between 18% and 32% of γ-carboxylated GRP at 10 μM K<sub>1</sub> when compared to wt in our in vitro assay (Figure 1a and Table 1). Levels of γ-carboxylated MGP varied between 2.9% and 102% for all these co-expressed GGCX variants (Figure 1a and
The skin phenotype is caused by the biallelic deficiency to γ-carboxylate GRP. (a–c) In vitro γ-carboxylation, dose–response curves of GRP and MGP measured by ELISA. The y-axis represents normalized γ-carboxylation (%) to wt. The x-axis represents $K_1$ concentrations (0.1–100 μM). Error bars depict SD of triplicate measurements of $n=3$ experiments. In each figure dose–response curve for GGCX wt is depicted in black. (a) In vitro γ-carboxylation, dose–response curves of GRP and MGP with respect to pathogenic GGCX variant reported in patients having a PXE-like phenotype. These variants were found to be compound heterozygous, where the other allele harbors a nonsense or loss-of-function variant. Compound heterozygous pathogenic variants are represented by the same color, with each allele variant represented in solid and dotted lines. (b) In vitro γ-carboxylation, dose–response curves of GRP and MGP with respect to variants reported to be compound heterozygous in a patient with a mild skin phenotype. (c) In vitro γ-carboxylation, dose–response curves of GRP and MGP with respect to variants that did not respond well to $K_1$ administration. Patients harboring these pathogenic variants are expected to develop a skin phenotype later in life.
| Reported data from previous studies | In vitro data from the present study |
|------------------------------------|------------------------------------|
| **GGCX variants**<sup>cDNA annotation</sup> | **GGCX variants**<sup>protein annotation</sup> | **Age (Age of onset)** | **Genotype** | **Non-hemostatic phenotype/s** | **Non-hemostatic phenotype/s** | **VKORC1c.-1639** | **Ref.s** | **γ-carboxylated GRP [%] at 10 µM K<sub>i</sub>** | **γ-carboxylated MGP [%] at 10 µM K<sub>i</sub>** | **γ-carboxylated BGLAP [%] at 10 µM K<sub>i</sub>** | **Comments** |
| GGCX variants | GGCX variants | | | | | | | | | | |
| c.247C>T | p.Arg83Trp | 48 years | CH | PXE-like phenotype | 26.2% | 31.7% | 15.8% | Li, Schurgers, et al. (2009) | Skin hyper-laxity |
| c.1120C>T | p.Gln374Ter | (27 years) | | | | | | | | | |
| c.1610G>C | p.Gly537Ala | 46 years | CH | PXE-like phenotype | 18.4% | 102% | 155.8% | Vanakker et al. (2007) | Skin hyper-laxity |
| c.1120C>T | p.Gln374Ter | 44 years | | | | | | | | |
| c.763G>A | p.Val255Met | 16 years | CH | PXE-like phenotype | 30.7% | 10.2% | 57.3% | Li, Grange, et al. (2009) | Skin hyper-laxity |
| c.899C>T | p.Ser300Phe | | | Cardiac abnormality | | | | | |
| c.1672G>A | c.896T>C | 40 years | CH | PXE-like phenotype | 31.7% | 18.2% | 65.8% | Vanakker et al. (2007) | Skin hyper-laxity |
| c.1211A>G | p.His404Pro | 47 years | CH | Mild skin symptom + Cardiac abnormality | 34.8% | 8.9% | 0.4% | Watzka et al. (2014) | Fragmentation of elastic fiber |
| c.1454G>C | p.Arg485Pro | | | | | | | | | |
| c.248G>C | c.248G>C | 3 years | HMZ | Facial Dysmorphism + Septal defect | 12.6% | 12.6% | 34% | Watzka et al. (2014) | Expected to develop PXE-like phenotype |
| c.248G>C | c.248G>C | | | | | | | | | |
| c.1181T>G | p.Leu394Arg | 5 months, newborns | HMZ | - | 33.3% | 36.1% | 0% | Brenner et al. (1998) | Expected to develop mild skin phenotype |
| c.1181T>G | p.Leu394Arg | | | | | | | | | |
| c.1479G>T | p.Trp493Cys | 1 year | CH | - | 2.3% | 81.5% | 55.8% | Darghouth et al. (2009) | Expected to develop PXE-like phenotype |
| c.2110C>T | p.Arg704Ter | | | | | | | | | |
| c.1502G>C | p.Trp501Ser | 7 days | HMZ | - | 2% | 72.8% | 55.8% | Spronk et al. (2000) | Expected to develop PXE-like phenotype |
| c.1502G>C | p.Trp501Ser | | | | | | | | | |

Note: This table summarizes previously reported VKCFD1 patients with skin symptoms. For patients with a PXE-like phenotype a biallelic reduction of GRP was detected (highlighted in red). Young VKCFD1 patients with markedly reduced levels of γ-carboxylated GRP are expected to develop a PXE-like phenotype later in life (highlighted in blue). –, not analyzed.
3.3 | Markedly reduced levels of γ-carboxylated MGP are crucial but not exclusive for causing skeletal defects

Five VKCFD1 patients were reported with skeletal dysmorphologies. A Keutel syndrome-like phenotype was described for a patient that is compound heterozygous for GGCX:c.[248G>C];[248G>C] (p.(Arg83Pro);p.(Arg83Pro)), GGCX:c.[1181T>G];[1181T>G] (p.(Leu394Arg);p.(Leu394Arg)), GGCX:c.[479G>T];[2110C>T] (p.(Trp704Ter);p.(Trp704Ter)) and GGCX:c.[1502G>C];[1502G>C] (p.(Thr591Lys+Arg204Cys);p.(Thr591Lys+Arg204Cys)). These patients are currently infants (Brenner et al., 1998; Darghouth et al., 2009; Spronk et al., 2010; Watzka et al., 2014) and the earliest reported age of onset for developing a PEX-like phenotype in VKCFD1 patients was 18 years (Vilder et al., 2017).

3.4 | Cardiac abnormalities and the impact of γ-carboxylated GRP and MGP

Three patients with GGCX variants GGCX:c.[c.248G>C];[c.248G>C] (p.(Arg83Pro);p.(Arg83Pro)), GGCX:c.[850T>C];[944G>A] (p.(Ser284Pro);p.(Trp315Ter)), and GGCX:c.[1672G>A];[896T>C] (p.(Gly558Arg);p.(Phe299Ser)) were reported with severely reduced levels of γ-carboxylated MGP as patients harboring variants GGCX:c.[1672G>A];[896T>C] p.(Gly558Arg);p.(Phe299Ser), GGCX:c.[763G>A];[899C>T] p.(Val255Met);p.(Ser300Phe), or GGCX:c.[247C>T];[1120C>T] p.(Arg83Trp);p.(Gln374Ter) were reported with skeletal malformation at birth (Figure 2c and Table 2).
[1454G>C] (p.(His404Pro);p.(Arg485Pro)), and GGCX:c.[1610G>C];[1120C>T] (p.(Gly537Ala);p.(Gln374Ter)) developed subclinical atherosclerosis. All these patients harbor pathogenic GGCX variants, which showed reduced ability to $\gamma$-carboxylate GRP and MGP in our ELISA assay (Table S4).

Levels of $\gamma$-carboxylation ranging from 0% to 218% were detected for BGLAP, GAS6, PRGP1, and TMG4 (Table S2).

### 3.5 | hGGCX structural model characteristics

To understand the specific structural implication of the reported pathogenic variants, a threading-based GGCX in silico model was generated. In the simulation-equilibrated model, the majority of the C-terminal region from 420 to 758 amino acid (aa) localized towards the ER luminal side including the propeptide binding site (495–513 aa; Parker et al., 2014; Figure S2). Our model has multiple transmembrane domains with a distribution of both proper helices (116–134, 240–262, 274–292, 362–377 aa) and molten globule regions (95–115, 394–419 aa) resembling loosely packed helical structure, which also bears the glutamate binding site (393–404 aa; Figure S2; Mutucumarana et al., 2000, 2003).

In the absence of adequate structural templates and considering the large size of GGCX, our in silico model, albeit entirely hypothetical reflects the best possible structural representation of GGCX protein that can be achieved presently. Reported GGCX variants are heterogeneously distributed throughout this in silico model (Figure S3).

**FIGURE 2** Markedly reduced levels of $\gamma$-carboxylated MGP are crucial but not exclusive for causing skeletal defects. (a–c) In vitro $\gamma$-carboxylation, dose–response curves of GRP, MGP, and BGLAP measured by ELISA. The $\gamma$-axis represents normalized $\gamma$-carboxylation (%) to wt. The x-axis represents $K_1$ concentrations (0.1–100 µM). In each figure dose–response curve for GGCX wt is depicted in black. Compound heterozygous pathogenic variants in one particular patient are represented by the same color with each allele variant in solid and dotted lines. Error bars depict SD of triplicate measurements of $n = 3$ experiments. (a) In vitro $\gamma$-carboxylation, dose–response curves with respect to variants found in patients having skeletal defects. (b) In vitro $\gamma$-carboxylation, dose–response curves for a variant reported in a patient having facial dysmorphologies, whose mother had severe hyperemesis gravidarum. The second allele harbors a nonsense variant. (c) In vitro $\gamma$-carboxylation, dose–response curves with respect to variants that showed low $\gamma$-carboxylated MGP values and the patient harboring these variants did not develop facial dysmorphologies.
| GGCX variant | cDNA annotation | GGCX variant | protein annotation | Age (age of onset) | Genotype | Non-hemostatic phenotype(s) | VKORC1c. - 1639 Refs. | In vitro data from the present study | Comments |
|--------------|----------------|--------------|--------------------|-------------------|----------|-----------------------------|----------------------|-----------------------------|----------|
| c.248G>C     | p.Arg833Pro    | c.248G>C     | p.Arg833Pro        | 3 years           | HMZ      | Facial Dysmorphism + Septal defect | AA Watzka et al. (2014) | γ-carboxylated GRP [%] at 10 µM K<sub>1</sub> 12.6% | 26.5% 34% |
| c.458A>G     | p.Asp153Gly    | c.521T>G     | p.Met174Arg        | 4 months          | CH       | Keutel syndrome-like phenotype | J.-K. Tie et al. (2016) | γ-carboxylated MGP [%] at 10 µM K<sub>1</sub> 93.5% 0.8% | 38.3% 1.2% 81.5% 0% |
| c.850T>C     | p.Ser284Pro    | c.944G>A     | p.Trp315Ter        | 13 years          | CH       | Midfacial hypoplasia + Septal defect | AA Watzka et al. (2014) | γ-carboxylated BGLAP [%] at 10 µM K<sub>1</sub> 80.1% 63.2% | – 100.2% 63.2% Mother had hyperemesis gravidarum |
| c.469T>C     | p.Trp157Arg    | (c.91G>A) +p.Thr591Lys | 2 years          | CH       | Developmental delay and stunted growth + Septal Defect | Darghouth et al. (2006) | γ–carboxylated MGP [%] at 10 µM K<sub>1</sub> 44.1% 31.8% | 58% 23.8% 45.1% 0% |
| c.610C>T     | p.Arg204Cys    | c.610C>T     | p.Arg204Cys        | 11 years          | CH       | Midfacial hypoplasia          | GA Watzka et al. (2014) | γ–carboxylated MGP [%] at 10 µM K<sub>1</sub> 67.2% 45.5% | 54.7% 45.5% 45.5% |
| c.1672G>A    | p.Gly558Arg    | p.Hep299Ser  | 40 years          | CH       | PXE-like phenotype            | Vanacker et al. (2007) | γ–carboxylated MGP [%] at 10 µM K<sub>1</sub> 31.7% 0% | 18.2% 1% 65.8% 0% No skeletal phenotype although low level of γ–carboxylated MGP |
| c.763G>A     | p.Val255Met    | p.Ser300Phe  | 16 years          | CH       | PXE-like phenotype + Cardiac abnormality | Li, Grange, et al. (2009) | γ–carboxylated MGP [%] at 10 µM K<sub>1</sub> 30.7% 1.5% | 10.2% 2.9% 57.3% 0% No skeletal phenotype although low level of γ–carboxylated MGP |
| c.247C>T     | p.Arg833Trp    | p.Gln374Ter  | 48 years          | CH       | PXE-like phenotype            | Li, Schurgers, et al. (2009) | γ–carboxylated MGP [%] at 10 µM K<sub>1</sub> 26.2% 31.7% | 15.8% | No skeletal phenotype although low level of γ–carboxylated MGP |

Note: This table summarizes the previously reported five VKCFD1 patients with skeletal defects. For some of these patients reduced values of γ-carboxylated MGP were detected in our in vitro assay. This table also includes variants with markedly reduced γ-carboxylated MGP levels although patients harboring these variants were not reported with skeletal defects (in black). –, not analyzed.
3.6 Identification of the vitamin K hydroquinone binding site in GGCX

Since no knowledge exists regarding KH2 binding, we docked the KH2 ligand on our simulation equilibrated model that revealed 29 docking poses (Figure S4). Out of these, only the docking pose 6 had the correct orientation of KH2 (with respect to membrane embedding) and is proximal to known active site residue Lys218 (Rishavy et al., 2006). Therefore, this docking pose was chosen for further analysis, in which residue Phe299 undergoes both Pi-Pi and hydrophobic interaction with the KH2 head group (Figures 3a,b and S5). Hence, the substitution of phenylalanine at position 299 with a polar serine residue can be expected to disrupt its interactions with KH2, resulting in loss of function (Figure 3f). To evaluate the effect of GGCX:p.(Ser300Phe) and the loss-of-function variant GGCX:p.(Met174Arg) on GGCX structure and KH2 binding, variant models were simulated. The production phase of GGCX:p.(Ser300Phe) mutated complex’s simulation showed a higher root mean square deviation (RMSD) for receptor and ligand movement when compared to wt (Figure 3c). The substitution of serine to phenylalanine breaks a small helix between residues 299–300, disturbing the spatial orientation and Pi-Pi interaction of Phe299 to the KH2 head group, thereby leading to markedly decreased values of γ-carboxylation for all investigated VKD proteins. The production phase for the simulation of the GGCX:p.(Met174Arg) mutated docked complex also showed an increase in RMSD, although not as high as GGCX:p.(Ser300Phe) (Figure 3f). We propose that GGCX:p.(Met174Arg) most likely alters the structural stability of GGCX which is in line with previous findings (J.-K. Tie et al., 2016), rather than interfering with KH2 binding. Further immunofluorescent (IF) staining confirmed that GGCX:p.(Phe299Ser) and GGCX:p.(Ser300Phe) were colocalizing to the ER similar to wt GGCX with a global PCC of +0.81 and +0.83, respectively (Figure 3d,e). GGCX:p.(Met174Arg) co-localizes to the ER with a significantly reduced global PCC of +0.69 when compared to wt (Figure 3e). The nonsense variant GGCX:p.(Trp315Ter) served as negative control and showed no expression. The positive control GGCX:p.(Lys218Ala), where the active site is mutated results in loss-of-function but is expressed as high as wt and co-localizes in the ER (Figures 3e and S6).

4 DISCUSSION

Here, we are giving evidence, which of the non-hemostatic VKD proteins could be involved in the development of non-hemorrhagic phenotypes in VKCFD1 patients and how the corresponding pathogenic GGCX variants are responding to vitamin K administration. These findings will lead to optimized treatment and might prevent in the future the development of non-hemorrhagic pathologies in patients harboring GGCX variants.

The non-hemorrhagic phenotypes in VKCFD1 summarize skin laxity, skeletal and cardiac abnormalities (Vilder et al., 2017). However, until now it remained elusive which under-carboxylated VKD protein causes the corresponding phenotype. Notably, we found that markedly reduced ability of GGCX variants to γ-carboxylate GRP in vitro is associated with a PXE-like phenotype reported in four VKCFD1 patients. The substantial defect to γ-carboxylate GRP by GGCX has to be biallelic for developing a PXE-like phenotype. The monoallelic disability to γ-carboxylate GRP is not leading to a skin phenotype as reported for all heterozygous family members (parents and siblings) of VKCFD1 patients with a PXE-like phenotype (Table S5). Notably, two VKCFD1 patients harboring GGCX variants, which lead to only monoallelic disability to γ-carboxylate GRP did not show skin symptoms at the age of 18 and 20 years [GGCX:c.[1595T>C];[521T>G] p.(Ile532Thr);(Met174Arg) and GGCX:c.[850T>C];[944G>A] p.(Ser284Pro);(Trp315Ter), Table S3]. The GGCX variants of the functional allele reported in these patients exhibited ~80% ability to γ-carboxylate GRP in our in vitro assay, which further indicates that moderate levels of γ-carboxylated GRP might prevent the development of a PXE-like phenotype. However, it has to be further observed if these patients will acquire a skin phenotype at an older age.

To our knowledge, this is the first study showing that the γ-carboxylation status of GRP contributes to the development of PXE-like phenotype in VKCFD1 patients. Although, no skin phenotype was reported in Grp−/− mice, it has to be considered that these mice were examined in the embryonic state, where no skin phenotype is expected (Eitzinger et al., 2012). In line with our findings, a study by Viegas et al. (2009) has shown that Grp is present in the epidermis and dermis levels of human skin and is highly expressed in the fibroblasts of the papillary and reticular dermis. Moreover, Viegas et al. (2014) showed that γ-carboxylated GRP is the predominant form in fibroblasts from healthy controls, whereas uncarboxylated GRP was found in skin cancer samples. However, further studies are needed to evaluate the importance of GRP with respect to skin calcification in Grp−/− mouse models and patient-derived samples.

Although the majority of the pathogenic GGCX variants identified in patients with a PXE-like phenotype exhibited substantially lower γ-carboxylation of MGP when compared to wt values, the variant GGCX:p.(Gly537Ala) showed similar ability to γ-carboxylate MGP as wt (Table 1). Therefore, under γ-carboxylated MGP might contribute to the severity of skin laxity. However, under γ-carboxylated MGP is not exclusively seen in all patients with a PXE-like phenotype. Since it was demonstrated by other studies that MGP act together with GRP in smooth muscle cells, there is the possibility that low levels of γ-carboxylated GRP might negatively regulate levels of γ-carboxylated MGP within the tissue (Viegas et al., 2015; Willems et al., 2018). Due to the markedly reduced levels of γ-carboxylated GRP obtained in our in vitro assay, we predict for three more patients the development of a PXE-like phenotype. Therefore, under γ-carboxylated GRP might contribute to the development of a PXE-like phenotype. We observed in our in vitro data that administration of high doses of vitamin K is not increasing γ-carboxylated GRP to substantial levels for GGCX variants reported to cause the PXE-like phenotype (Figure 1a). Therefore, life-long administration of high doses of vitamin K might improve but not prevent the development of a skin phenotype. However, these patients...
FIGURE 3  (See caption on next page)
might be mediated in future by the nanotechnology-based method of Viegas et al. (2019), where human γ-carboxylated GRP is loaded into extracellular vesicles. This recent and smart approach was invented to inhibit vascular calcification, which might have a high potential to treat ectopic calcification of skin, too.

Besides the PXE-like phenotype, skeletal malformations including facial dysmorphologies were observed in a low number of VKCFD1 patients. As loss-of-function variants in MGP lead to Keutel syndrome, which is an autosomal recessive disorder characterized by midfacial hypoplasia and other skeletal malformations (Munroe et al., 1999), it was expected that GGCX variants failing to γ-carboxylate MGP lead to skeletal defects. J.-K. Tie et al. (2016) stated in their work that under-carboxylated MGP caused by GGCX:p.(Asp153Gly) and p.(Met174Arg) is responsible for the Keutel syndrome-like phenotype in a patient carrying GGCX:c.[521T>G +974G>A];[458A>G] (p.(Met174Arg+Arg325Gln);p.(Asp153Gly)). We confirmed their findings for the variants harbored in this patient with undetectable levels of γ-carboxylated MGP when co-expressed with GGCX:p.(Met174Arg) and with levels of ~38% γ-carboxylated MGP when co-expressed with GGCX:p.(Asp153Gly). In line with these findings, low levels of γ-carboxylated MGP below 27% were detected for another affected VKCFD1 patient diagnosed with facial hypoplasia harboring the genotype GGCX:c.[248G>C];[248G>C] p.(Arg83Pro);p.(Arg83Pro).

However, two more patients were reported with skeletal defects and the genotype GGCX:c.[610C>T];[610C>T] (p.(Arg204Cys);p.(Arg204Cys)) or GGCX:c.[469T>C];[91G>A; 1772C>A] (p.(Trp157Arg);p.(Asp31Asn+Thr591Lys)). The corresponding pathogenic GGCX variants did not display markedly reduced levels of γ-carboxylated MGP in our in vitro data. This indicates that reduced levels of γ-carboxylated MGP are not the only causative factor for developing skeletal defects in VKCFD1 patients. In line with these results, there are also VKCFD1 patients reported, who did not develop congenital skeletal malformations, although they harbor GGCX variants that exhibit markedly reduced levels below 26% of γ-carboxylated MGP in our in vitro assay (Table 2). Another argument is the fact that the siblings that carry the same GGCX genotype (GGCX:c.[610C>T];[610C>T]) showed a similar hemorhagic phenotype, but only one of them shows skeletal malformations, whereas the other does not. Altogether, this demonstrates that additional factors like other genes or epigenetic changes contribute to the development of skeletal malformations (Tiago et al., 2016). Therefore, we conclude that the biallelic reduction of γ-carboxylated MGP by GGCX variants is associated with the development of skeletal defects in some cases, but not exclusive for causing these defects in VKCFD1 patients.

As the skeletal phenotype is a congenital defect that is developed during embryogenesis, we suspect that the maternal genotype and vitamin K uptake during pregnancy contributes to the development of facial malformations as well. Indeed, nutritional uptake by the mother can affect the degree of malformation, which was shown by the study of Lanham et al., where *Mgp<−/−* mice, whose mothers were fed on a high-fat diet during pregnancy showed increased bone parameters as bone length, volume, and surface compared to littermates, whose mothers were fed on a control diet. This suggests a maternal nutrient transfer is influencing the outcome of skeletal birth defects in *Mgp<−/−* mice. Moreover, Lanham et al. (2018) also showed that GGCX expression is higher in *Mgp<−/−* mice, whose mothers were fed on a high-fat diet when compared to control littermates suggesting that GGCX expression could be regulated through substrate availability. Altogether, this indicates that the nutritional uptake of vitamin K during pregnancy might modulate the severity of those birth defects in humans as well. Therefore, we assume that the severe hypemesis gravidarum with a weight loss of seven kg within the first trimester from the mother of the patient carrying GGCX:c.[850T>C];[944G>A] (p.(Ser284Pro);p.(Trp315Ter)) had a significant additional impact on the development of the patient’s midfacial hypoplasia. Although this patient carries one functional allele, where GGCX:p.(Ser284Pro) exhibits nearly normal level of γ-carboxylated MGP at high vitamin K levels, this patient was born with midfacial hypoplasia. This together with the findings of Lanham et al. suggests that nutritional uptake during pregnancy also modulates the severity of the congenital skeletal defects in VKCFD1 patients.

Furthermore, we agree with Watzka et al. (2014) that the promoter polymorphism of VKORC1 (NG_011564.1: g.3588G>A) might modulate the severity of VKCFD1 phenotypes, especially with respect to skeletal malformations. The global minor allele frequency...
NG_011564.1:g.3588G>A (VKCFD1 patients with facial hypoplasia are homozygous for the rare GMAF of NG_011564.1:g.3588G>A (rs9923231) is 0.35563 (T). GHOSH ET AL. it could influence the severity of both hemorrhagic and non-hemorrhagic phenotypes in VKCFD1 patients due to the reduced capacity to recycle vitamin K through VKORC1. Therefore, we recommend genotype VKCFD1 patients also for the promoter polymorphism in VKORC1 in future to evaluate the efficiency of therapy. Patients with the weak NG_011564.1:g.3588G>A (VKORC1:c.-1639 AA) genotype might develop more severe phenotypes and thus need higher K treatment.

In VKCFD1 patients with a cardiac phenotype, we observed for the corresponding reported pathogenic GGCX variants a reduced ability to γ-carboxylate GRP and MGP (Table S3). However, not all patients carrying pathogenic GGCX variants causing reduced levels of γ-carboxylated GRP and MGP were reported with cardiac defects. As GAS6 was shown to play a role in cardiac pathology, we anticipated that this protein might be undercarboxylated in these patients (McShane et al., 2019). However, in our in vitro data, we did not find a correlation between GAS6 and any other analyzed VKD protein with GGCX variants identified in VKCFD1 patients with cardiac defects. Here, we assume again that the nutritional uptake and the lifestyle affect the aging phenotype as atherosclerosis, where patients with low vitamin K uptake have a higher risk to develop a cardiac phenotype. Furthermore, it is possible that under γ-carboxylation of other proteins, which were not analyzed in the present study is causing cardiac abnormalities in VKCFD1 patients.

With respect to the structure of GGCX, our in silico model allowed us to predict the vitamin K binding site. Two pathogenic GGCX variants directly affecting this binding site (GGCX:p.(Phe299Ser) and GGCX:p.(Ser300Phe)) resulted in loss-of-function for γ-carboxylation of all analyzed VKD proteins. Both GGCX variants were also found in another study to cause loss-of-function or diminished ability to γ-carboxylate VKD clotting factors (Hao et al., 2021). Hao et al. also showed that these two residues show reduced vitamin k epoxidation when mutated. Although our GGCX in silico model has limitations due to its unique structure, we propose that variants GGCX:p.(Phe299Ser) and GGCX:p.(Ser300Phe) disrupt the vitamin K binding, which might take place in this region. However, future studies are required to solve the X-ray crystal structures of GGCX for identifying the exact vitamin K binding domain.

Even though non-hemostatic VKCFD1 phenotypes are rare, this study highlights the need for GGCX to sufficiently γ-carboxylate GRP and MGP for maintaining physiological calcification. Our data will help to further understand the diversity of known and potentially unknown VKCFD1 phenotypes. The outcome of birth defects could be potentially improved when mothers carrying an offspring with a GGCX defect are administered vitamin K during pregnancy. Personalized medication with γ-carboxylated VKD proteins as for example γ-carboxylated GRP to treat non-hemorrhagic phenotypes may represent a promising strategy in future as vitamin K administration is not improving γ-carboxylation for VKD proteins by specific GGCX variants and thus might not prevent the development of all phenotypes.

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CONFLICT OF INTERESTS
The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS
Experimental design (Katrin J. Czogalla-Nitsche, Jens Müller, and Suvoshree Ghosh), generation of GGCX−/− cells (Veit Hornung, Klara Höning, and Katrin J. Czogalla-Nitsche) data collection (Suvoshree Ghosh and Katrin Kraus), data analysis & figure production (Suvoshree Ghosh and Katrin Czogalla-Nitsche), protein modeling (Suvoshree Ghosh, Arijit Biswas, and Francesco Forin) manuscript drafting and editing (Suvoshree Ghosh, Katrin J. Czogalla-Nitsche, Heike Singer, Veit Hornung, Klara Höning, Matthias Watzka, and Johannes Oldenburg).

DATA AVAILABILITY STATEMENT
All γ-carboxylation data were obtained from triplicate measurements of n = 3 experiments and are summarized in Tables 1 and 2. For Figures 1–3, γ-carboxylation values are given in the Supporting Information. If further data are required, data are available on request.

WEB RESOURCES
https://zhanglab.ccmb.med.umich.edu/I-TASSER/
https://variantvalidator.org/

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