# Metabolism of vitamin $D_3$ in the placental tissue of normal and preeclampsia complicated pregnancies and premature births

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**Summary**

The aim of this study was to analyze the hormonal basis for low $1,25(OH)_2D_3$ circulating levels in patients with preeclampsia and/or preterm deliveries. The activity and expression of the 1αOHase, 25-OHase, 24-OHase and VDR in the placental tissue of normal pregnancies, preeclampsia-complicated pregnancies and premature births were investigated.

The mRNA of the enzymes was detected in the placental tissue from preeclamptic pregnancies and compared to those of normal placental tissue. Real time PCR analysis showed a significant increased 1α-OHase gene expression in preeclamptic patients, and the gene expression of 24-OHase was significantly decreased. With regard to the 25-OHase the median value of the normal placental tissue was significantly higher than in the placental tissue of preeclamptic patients.

The real time analysis of all target genes also showed significant differences in normal placental tissue compared to placental tissue from premature births (VDR: $p = 0.041$; 1α-OHase: $p = 0.013$; 24-OHase: $p = 0.007$; 25-OHase $p = 0.027$).

Our observation of reduced VDR expression on mRNA level in placental tissue indicates a possible dependence of the modulation of VDR expression from proliferation and differentiation processes. It can be speculated whether the down-regulation of VDR in the examined placenta cells was the result of an altered production of calcitriol by these cells.

We found a significantly higher 1α-OHase-expression in the placental tissue of pregnant women with preeclampsia or preterm birth compared to healthy pregnant women, whereas the expression of 25-OHase was significantly reduced. These results correlate with other studies and support the significance of the placenta regarding metabolism malfunctions as they were observed in the calcium metabolism for preeclampsia. That a placenta with preeclampsia expresses less 1α-OHase-mRNA and shows less 1α-OHase-activity than in placental samples of inconspicuous placentae, can be granted as a specific alteration in the placental ability to synthesize adequate amounts of $1,25(OH)_2D_3$.

*Key words:* Vitamin D3; VDR; 1α-hydroxylase; Placenta; Preeclampsia.

## Introduction

The hormonally active metabolite of vitamin $D_3$ 1,25-dihydroxyvitamin D ($1,25(OH)_2D_3$) plays a main role in mineral homeostasis by facilitating calcium and phosphate absorption in the bowel and by modulating bone cell development and function [1]. However, it was shown that vitamin D influences a wide range of physiological functions not linked to calcium homeostasis, e.g. anti-proliferative and immunosuppressive effects have been proven [2-4]. It is generally accepted that the anti-proliferative effect is the result of vitamin D receptor (VDR)-mediated action on the genome [5]. Its function depends primarily on the local availability of the ligand; vitamin $D_3$ has a strong affinity for VDR.

Two principal enzymes are involved in the formation of circulating $1,25(OH)_2D_3$ from vitamin D; the hepatic microsomal or mitochondrial vitamin D-25-hydroxylase (25-OHase) and the renal mitochondrial 25-hydroxyvitaminD3-1α-hydroxylase (1α-OHase) for vitamin D and $25(OH)D_3$, respectively [6, 7]. The first observation leading to the establishment of an extra-renal source of 1α-OHase was in pregnant rats, which showed reduced, but not completely eliminated serum concentrations of $1,25(OH)_2D_3$ [8]. It has been reported that decidua cells synthesize $1,25(OH)_2D_3$ during pregnancy [9] and $1,25(OH)_2D_3$ has also been produced in vitro by human and rodent placental trophoblast cells [8, 9]. However, a consistent and detectable production of $1,25(OH)_2D_3$ by these cells could not be demonstrated [10, 11]. Besides the synthesis of $1,25(OH)_2D_3$ the expression of VDR was proven [12]. Moreover, $1,25(OH)_2D_3$ activates its own breakdown by stimulating transcription of the 24-hydroxylase (24-OHase) gene; 24-OHase inactivates the vitamin D metabolites $25(OH)D_3$ and $1,25(OH)_2D_3$.

In view of the fact that reproduction in females is markedly diminished in states of vitamin D deficiency [13], it was postulated that local synthesis of $1,25(OH)_2D_3$ might play a role in implantation and/or placentation [14]. It was shown that the expression of 1α-OHase in decidua is ~1000-fold higher in the first and second trimester in comparison to the third trimester. The expression of 1α-OHase in placenta tissue was ~80-fold higher in the first two trimesters than in the third. Parallel analyses of VDR indicated that the highest levels of expression of the receptor occurred in first trimester decidua [14].

Patients with pregnancy-induced hypertension or preeclampsia show abnormalities in calcium metabolism [15-17]. Preeclampsia is associated with low circulating levels of $1,25(OH)_2D_3$ in the maternal and umbilical cord

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compartments [12]. It is possible that alterations in calcium metabolism could occur at the level of the feto-placental unit. Placental tissue expresses the mitochondrial cytochrome $p_{450}$1α-OHase (Cyp 27b1) gene, which is in accord with the capability of the placenta to produce $1,25(OH)_2D_3$ [18]. A low level of $1,25(OH)_2D_3$ in maternal blood is a risk factor for preterm delivery [19]. Preeclampsia is associated with preterm delivery as well, but the pathomechanism is still unclear.

The aim of this study was to analyze the hormonal basis for low $1,25(OH)_2D_3$ circulating levels in patients with preeclampsia and/or preterm deliveries. The activity and expression of 1αOHase, 25-OHase, 24-OHase and VDR in the placental tissue of normal pregnancies, preeclampsia-complicated pregnancies and premature births were investigated.

## Materials and Methods

Placentae were obtained from pregnant women after spontaneous delivery or Caesarean sections. Tissues were immediately snap-frozen in liquid nitrogen (-196°C) and stored at -70°C until further treatment. Histological examination by a certified pathologist was obtained.

*RNA-isolation*

Tissue was coarsely minced and mixed with TRI-reagent (Sigma, product no. T9424). The cell suspension was homogenized, mixed with 400 µl chloroform for 15 sec and stored for 15 min at room temperature. After this incubation period the suspension was centrifuged at 10,000 rpm for 15 min at 4°C. The upper band, containing the RNA, was removed, resuspended with 500 µl isopropanol and again centrifuged at 13,000 rpm for 15 min at 4°C. The RNA was washed once with 1 µl ethanol, resuspended with vortex and recentrifuged at 13,000 rpm for 5 min at 4°C. The supernatant was removed and the RNA-pellet dried for 5-10 min at room temperature. Afterwards it was resuspended in 100 µl of distilled water, aliquoted and frozen at -70°C. The RNA should have a 260/280 λ quotient of ≥ 1.7. To verify the RNA-isolation the samples were made with an ethidium bromide agarose gel with glyceraldehydes-3-phosphate dehydrogenase (GAPDH).

*Complementary DNA (cDNA) synthesis*

Total RNA (7 µl) and 1 µl random primer were mixed and heated at 65°C for 5 min. The following components were added: 5 µl 5* RT buffer, 2.5 µl dNTP4-mix 2.5 mM, 0.5 µl MMLV-RT (200 U), 1 µl Rnasin (40 U) and 8 µl $H_2O$. The suspension was incubated at 37°C for one hour and afterwards placed on ice.

To calculate the efficiency of the first strand synthesis 5 µl from the reaction were removed and added to a tube containing 5 µl 10*taq buffer, 4 µl dNTP, 1 µl primer (0,5 pmol), 2.5 µl taq polymerase and 31.5 µl $H_2O$. Conventional PCR was done in a DNA Block Cycler (Perkin Elmer 2400, Foster, CA, USA) according to manufacturer's instructions.

10 µl of the probe ran on a 1% agarose gel with DNA ladder (1 µg/ µl, 123 bp DNA-ladder, SIGMA, Missouri, USA). The gel was made with 10 µl of ethidium bromide stock solution (10 mg/ml) per 100 ml agarose solution. The gel ran under a voltage of 60 V for one hour. The results were analysed under a UV-lamp.

*Real-time reverse transcriptase polymerase chain reaction (LightCycler)*

The expressions of vitamin D receptor (VDR), 1α-, 24- and 25-OHase were analyzed using a real-time quantification method (LightCycler) according to the manufacturer's recommendations (Roche Diagnostics, Grenzach, Germany). The target gene was normalized to the housekeeping gene GAPDH as an internal standard using specific hybridization probes (LCRed 640 and fluorescein) for the target gene and GAPDH as a detection format. Purified PCR products were used as external standards (High Pure PCR Purification Kit, Roche Diagnostics). External standards were produced with a ten-fold serial dilution of a PCR product of the cDNA HaCaT cell line, which highly expresses VDR and the enzymes of vitamin D metabolism. The PCR sequence-specific primers used were as follows: for VDR: Forward: 5´-CCAgTTCgTgTgAATgATgg-3´, reverse: 5´-gTCgTCCATggTgAAggA-3´. For 1α-OHase: Forward: 5´-TgTTTgCATTTgCTCAgA-3´, reverse: 5´-CCgggAgAgCTCATACAg-3´. For the 24-OHase: Forward: 5´-gCAgCCTAgTgCAgATTT-3´; reverse 5´-ATTCACCCA-gAACTgTTg-3´. The primer for the 25-OHase was forward 5´-ggCAAgTACCCAgTACgg-3´ and reverse 5´-AgCAAATAgCTTCCAAgg-3´. For GAPDH the primers were: Forward: 5´-TTggTATCgTggAAggACTCA-3´. Reverse: 5´-TgTCATCATATTTggCAggTTT-3´.

cDNA (2 µl) was added to 18 µl master mix containing (specific for primers) 9.6 µl/ 9.8 µl/ 10.4 µl $H_2O$dd, $MgCl_2$ (2.5 mM for 1α-OHase, 3mM for GAPDH and 25-OHase, 4 mM for VDR and 24-OHase), primer pairs 0.5 µM 2 µl and 4 µl for 1α-OHase, respectively, and hybridization probes 0.25 µM (LCRed 640 and flourescein) 1 µl, respectively. The PCR was done according to manufacturer's instructions. Quantification of the target gene expression was obtained by direct comparison with external standards amplified in parallel reactions in the same run. The target load in the unknown samples was quantified by measuring the $C_t$ (threshold cycle) value and a standard curve to determine the starting target message quantity. The amplification efficiency of the target and housekeeping gene (GAPDH) did not differ > ± 0.05. Finally, the target gene/GAPDH ratio was calculated in order to normalize the data.

*Statistical analysis*

The non-parametric Mann-Whitney U test was used to evaluate the target gene/GAPDH ratios in the samples. Also the chi-square and the Kruskal-Wallis test were used.

## Results

The mRNA of 1α-OHase, 24-OHase and 25-OHase was detected in normal placental tissue and in placental tissue from preeclamptic pregnancies. Real time PCR analysis showed 1α-OHase gene expression in placental tissue from preeclamptic pregnancies that was significantly increased (p = 0.003) as compared to normal tissue (Figure 1). The gene expression of 24-OHase in placental tissue from preeclamptic patients was significant decreased (p = 0.006) as compared to normal tissue (Figure 2). With regard to the 25-OHase also a significant difference was shown: the median value of the normal placental tissue was significantly higher than in the placental tissue of preeclamptic patients (p = 0.038) (Figure 3).

The mRNA of VDR, 1α-OHase, 24-OHase and 25-OHase was also compared in normal placental tissue and

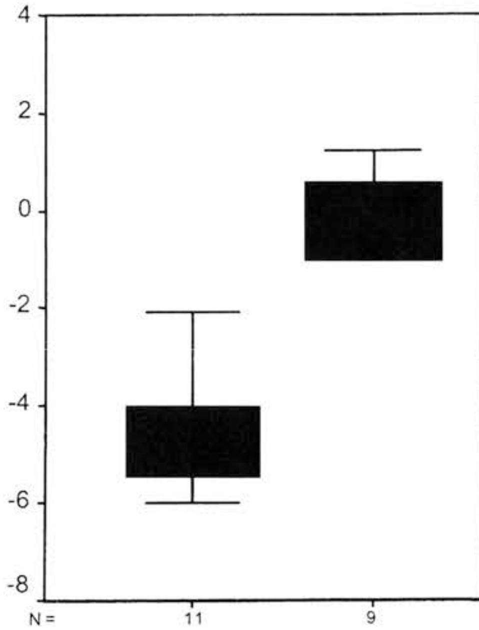


Figure 1. — Expression of 1αOHase in placental tissue of controls and preeclamptic patients.

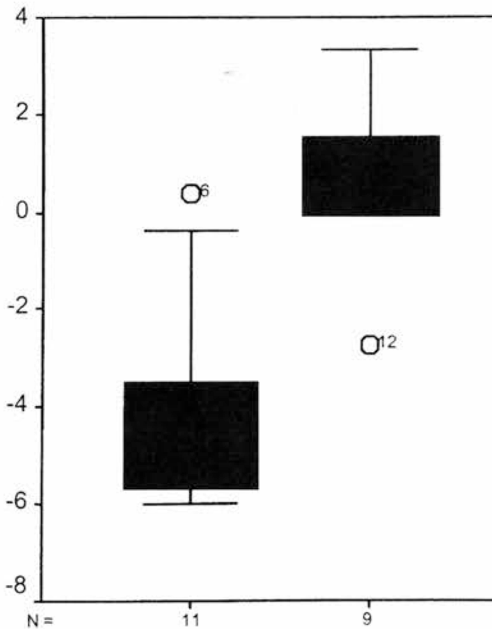


Figure 2. — Expression of 25-OHase in placental tissue of controls and preeclamptic patients.

in placental tissue from premature births (Table 1). Real time analysis showed significant differences in gene expression of all target genes in the placental tissue of women with premature birth compared to normal placental tissue (VDR: $p = 0.041$; 1α-OHase: $p = 0.013$; 24-OHase: $p = 0.007$; 25-OHase $p = 0.027$).

The HaCaT cell line served as a positive control for these experiments.

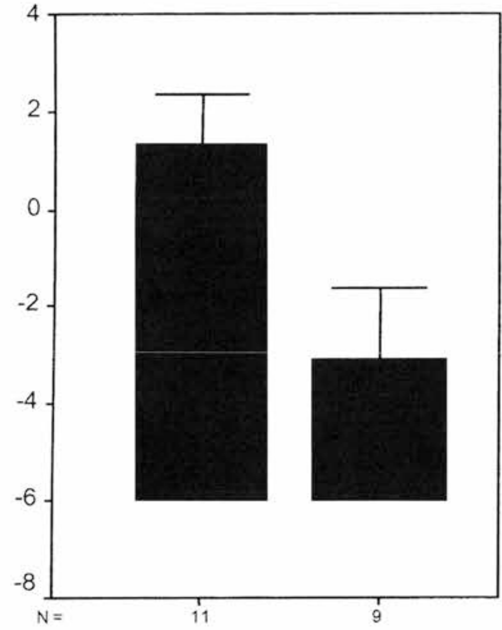


Figure 3. — Expression of 24-OHase in placental tissue of controls and preeclamptic patients

## Discussion

*Pathogenesis of premature birth and preeclampsia*

The incidence of preeclampsia is indicated with 3-5% in Germany [20]. In spite of numerous studies the pathophysiology has been investigated inadequately; an insufficient trophoblast invasion has been observed in the spiral arteries in the uterine musculature [21], which inhibits the desired vascular dilatation and therefore the physiological reduction of resistance in the placenta. The reduced placental perfusion entails a permeability dysfunction of the endothelium which leads to an overbalance of thromboxan compared to prostacyclin [21]. A general vasospasm and increased sensitivity for vasoactive substances such as angiotensin II cause pathological changes in the vessels, liver, kidneys and brain. The result is uteroplacental perfusion dysfunction [19, 20], which leads to intrauterine growth retardation and (partially iatrogenic) premature birth for the foetus and to eclampsia for the mother, respectively. Mother and child are affected by an increased risk of placental ablation.

Apart from preeclampsia and placenta insufficiency there are further risk factors for preterm deliveries: local infections can trigger premature labour pain or even premature rupture of the membranes. Damage in the cervix area (e.g. after operations), multiples and a low socioeconomic status are only a few causes for preterm delivery. Often neither the causes nor the pathomechanism are known.

There are indications that vitamin-D-metabolism influences fertility, preeclampsia and premature birth risk [23, 24]. Some of the changes seem to be caused intraplacentally. To gain insights into vitamin D3-metabolism in the placental tissue of women with preeclampsia and premature birth, mRNA expression of VDR and the enzymes of

placental tissue of healthy pregnant women were compared to preclamptic patients and those with preterm deliveries.

*VDR, 24-OHase*

The regulation of proliferation and differentiation in different cell types takes place among others through $1,25(OH)_2D_3$ and its receptor VDR [25], with VDREs found especially in genes which are involved in the cellular growth, differentiation, apoptosis, invasion and metastasis of cells [26]. The presence of VDR in the placental tissue supports the thesis that $1,25(OH)_2D_3$ is involved in the transportation process of calcium through the placenta [27] by up-regulating the VDR through an increase in stability on the mRNA and protein level. Some examinations show a close correlation between VDR concentrations and biological reactivity of the goal cells [28, 29].

We observed a significant decrease in expression of VDR on the mRNA level in the placental tissue of pregnant women with preeclampsia in comparison to the placental tissue sample of inconspicuous pregnant women whereas the expression of VDR in the placental tissue in case of preterm delivery was increased significantly. The expression of 24-OHase is significantly increased in placental tissue in cases of preeclampsia as well as in cases of preterm birth compared to the placental tissue of unsuspicious pregnant women. The different concentration of VDR on mRNA level could be the result of an altered $1,25(OH)_2D_3$ generation in the sense of a homologous regulation in the placenta of affected pregnant women. This appears interesting insofar as the effect of $1,25(OH)_2D_3$ with reference to differentiation and apoptosis induction is proven [25]. Cellular VDR expression seems to represent a function of the differentiation stadium. Our observation of reduced VDR expression on the mRNA level in placental tissue correlates with earlier examinations [18] and could mirror an altered differentiation pattern. This indicates a possible dependence of the modulation of VDR expression from proliferation and differentiation processes. It can be speculated whether the down-regulation of VDR in the examined placenta cells is the result of an altered production of calcitriol by these cells [30].

*1αOHase, 25-OHase*

In former studies it was found that the 1α-OHase from syncytiotrophoblast cells of preeclamptic patients showed one tenth of the activity of those from healthy women [18]. The results supported the significance of the placenta with respect to metabolism malfunctions as they were observed in the calcium metabolism for preeclampsia [31, 32]. A placenta with preeclampsia expresses less 1α-OHase-mRNA and shows less 1α-OHase-activity than placenta samples of inconspicuous placentae. This can be granted as a specific alteration in the placental ability to synthesize adequate amounts of $1,25(OH)_2D_3$.

On the other hand it has been verified that the trophoblasts of preeclamptic pregnant women differentiate the same way as those of healthy women to syncytiotrophoblasts and produce HCG [24], so that no defective cell differentiation seems to cause the low 1α-OHase-gene expression and enzyme differentiation.

The increase of the maternal serum with $1,25(OH)_2D_3$ is seen as a mechanism that increases calcium absorption during pregnancy [33]. It could be shown that placental tissue synthesizes the active metabolite of vitamin D by gene expression and activation of 1α-OHase [34].

To analyse the influence of $1,25(OH)_2D_3$-synthesis on the different stages of pregnancy, 1α-OHase-expression was measured in placenta with intrauterine growth retardation (IUGR) [14]. IUGR is characterized among others by abnormal placentation: the villous placenta is significantly less distinctive with reduced cytotrophoblast proliferation and reduced vascularisation. The 1α-OHase-mRNA-expression turned out to be up to 80 times higher in the first and second trimester than in the third trimester. It was deducted that the local $1,25(OH)_2D_3$-synthesis might play a role in the implantation or placentation. These results correlate with our studies, where we observed a significantly higher 1α-OHase-expression in the placental tissue of pregnant women with preeclampsia and preterm birth compared to healthy pregnant women, whereas the expression of 25-OHase was significantly reduced.

*Therapy*

A pharmacologic inhibition of 24-OHase activity or an intensification of 1α-OHase could be useful in the treatment of preeclampsia and premature birth to use the antiproliferative local activity of $1,25(OH)_2D_3$. The examined expression of the enzymes leads to the fact that the effects of $1,25(OH)_2D_3$ are defined less by the concentration in the serum but rather by the local synthesis. The synthesis itself is among others dependent on the offered amount of substrate. However the input of vitamin D is limited by its hypercalcaemic side-effects, thus future development of analogue and precursors of calcitriols remains to be seen.

## Conclusion

The metabolism of vitamin D3 in the placental tissue of preeclampsia-complicated pregnancies and premature births was found to be altered compared to normal pregnancies.

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