Cytochrome P450 metabolism of the post-lanosterol intermediates explains enigmas of cholesterol synthesis

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Cholesterol synthesis is a basic pathway believed to take place in virtually all mammalian cells. In this anabolic pathway the cholesterol molecule is built from acetate in over 30 reactions and enzymes from different protein families. The pathway is composed of the isoprenoid synthetic reactions forming squalene (the pre-squalene or pre-lanosterol part), which is well characterized1, and the post-squalene phase in which demethylations and reductions of sterol intermediates convert lanosterol to cholesterol. A detailed metabolic reaction network, with information available from the literature and the pathway databases (Kyoto Encyclopedia of Genes and Genomes (KEGG) (http://www.genome.jp/kegg/), BioCyc database collection (http://biocyc.org/), LIPID Metabolites And Pathways Strategy (LIPID MAPS) (http://www.lipidmaps.org/)), is shown in Supplementary Fig. 12–7. Initially the post-squalene pathway was divided into the Bloch and Kandutsch-Russell (K-R) branches8,9. In the Bloch branch, the final reaction to form cholesterol involves the conversion of desmosterol to cholesterol by sterol-Δ24-reductase (DHCR24); thus, all intermediates from lanosterol to desmosterol contain an unsaturated Δ24 bond. In contrast, in the K-R branch, DHCR24 acts earlier, on lanosterol; thus all intermediates from 24,25-dihydrolanosterol (DHL) to 7-dehydrocholesterol (7-DHC) contain a saturated side chain. Since DHCR24 can, in principle, metabolize any cholesterol synthesis intermediate from lanosterol on, the two branches are usually combined to produce a textbook cholesterol synthesis pathway4. A recent flux analysis of cholesterol synthesis surprisingly revealed that the K–R pathway is not used in tissues and cells in vivo10. Instead, a hybrid pathway exists where the transition from the Bloch to the K–R pathway depends on the tissue or cell type. Testes apply mainly the Bloch pathway, and the flux of intermediates drops from testis meiosis-activating sterol (T-MAS) to

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zymosterol. These findings suggested that a large fraction of sterols is diverted from the cholesterol pathway in the testis to produce other, yet unidentified sterols. Metabolism of lanosterol differs between liver and the testes, as shown in earlier work and confirmed by the recent flux analysis. We show here that intermediates of the Bloch and K-R branches leak from the pathway and can be metabolized by enzymes of the cytochrome P450 (CYP) superfamily in the testes of mice with defective post-lanosterol cholesterol synthesis. The leakage was proposed by a mathematical model in which the textbook cholesterol synthesis pathways failed to describe the experimental gene expression and sterol data. In the second iterative cycle, in vitro enzyme activity studies identified novel enzymes that catalyse sterol modifications outside the Bloch and K-R pathways, thus confirming the proposed in silico branches. The applied mouse model involves a knock-out for the transcription factor Crem, where males are infertile due to an arrest in spermatogenesis. Among other defects, the mice lack the germ cell-specific lanosterol-14 α-demethylase (Cyp51) mRNA producing the germine form of Cyp51 from the post-lanosterol cholesterol synthesis. T-MAS accumulates in the testes of normal mice due to cAMP responsive element modulator (CREM)-dependent transcriptional activation of Cyp51 and can be formed even in mature sperm. In this work we provide evidence of novel branching points in the post-lanosterol cholesterol synthesis and reveal new roles of CYP enzymes in cholesterol homeostasis.

Results

Gene expression in testis of Crem−/− mice. The relative expression levels of 15 cholesterogenic genes, Crem−/− compared to wild-types (WT), are shown in Fig. 1, with 13 of them being involved in the cholesterol synthesis pathway and two in post-cholesterol synthesis transformations of cholesterol (cytochrome P450-family-11 subfamily-A-polypeptide-1 (Cyp11a1) gene and cytochrome P450, family 27, subfamily A, polypeptide 1 (Cyp27a1) gene). Triangles (WT) and circles (Crem−/−) represent fold change of each individual mouse relative expression compared to average WT expression for each cholesterogenic gene separately. Five genes of the cholesterol synthesis pathway showed statistically significant higher expressions in Crem−/− compared to WT animals, Hmgcr (1.65-fold), Fdf1 (3.09-fold), Sqle (1.42-fold), Hsd17b7 (1.55-fold) and Elbp (1.81-fold), with p-values lower than 0.001, 0.01, 0.005, 0.001, and 0.001, respectively. Only two showed statistically significant lower expression levels, Cyp51 (0.39-fold) as the proposed direct target of CREM regulation and Dhcr7 (0.29-fold), with p-values < 0.001. Expression of the six remaining cholesterol synthesis genes (Lss, Dhcr24, Tm7sf2, Sc4mol, Nsdhl, and Sc5d) was unchanged. Expression of two genes involved in the cholesterol metabolism was also evaluated in Crem−/− compared to WTs. Cyp11a1 expression was increased (1.74-fold) while Cyp27a1 remained unchanged, with p-values of p < 0.01 and p = 0.11, respectively.

Cholesterol synthesis intermediates in testes of Crem−/− mice. Log10(μg/g testes wet weight) values for sterol intermediate concentrations are presented in Fig. 2 as boxplots, showing that lanosterol was significantly higher (p < 0.001, as expected due to the lower expression of Cyp51 gene, T-MAS (p < 0.001), lathosterol (p < 0.001), 7-DHC (p < 0.001) and desmosterol (p = 0.007) were significantly lower and the final product of the synthesis pathway, cholesterol, was significantly higher (p < 0.001) in mouse testes of 33 Crem−/− compared to 42 WT animals, adjusted on the bases of animal age and experiment date. Interestingly, lanosterol was highly accumulated (3.28-fold increase) while T-MAS (0.26-fold) and lathosterol (0.53-fold) show the largest drops in the Crem−/− testis. Desmosterol and 7-DHC, the immediate precursors of cholesterol were slightly (0.88-fold and 0.86-fold) diminished in the Crem−/− while cholesterol was slightly elevated (1.15-fold).

Simulation of the cholesterol synthesis network model proposed virtual enzymes that metabolize post-lanosterol sterols. The simulation of the initial (textbook) model (Fig. S1) showed significant differences between the simulated and actual metabolite levels. Stepwise modifications of the model were performed in order to correlate the experimental and simulated data (see Supporting Information). Initial model 0.
Figure 2. Cholesterol synthesis sterol concentrations. Boxplots represent log₁₀(µg/g testes wet weight) measurements of the sterols lanosterol, T-MAS (testis meiosis-activating sterol), lathosterol, 7-DHC (7-dehydrocholesterol), desmosterol, and cholesterol for each genotype (WT, wild-type; KO, Crem-/-) with associated p-values.
The initial (textbook) model was used without modifications and relative gene expressions were directly translated to the relative enzyme activities. The relative metabolite concentrations are presented in Fig. S2. The model did not describe the observed situation well because levels of several metabolites were much higher in the model. In the first model correction (model 1), the enzyme level changes were assumed to correlate perfectly with the corresponding mRNA levels for the late part of the cholesterol synthesis; however, levels of 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCGR) were optimized, as described in the Supplementary Information. These modifications resulted in an optimal fit of simulated and measured data for lanosterol, but the fit for other metabolites was as poor as in the initial model settings (Fig. S3). The resulting gene expressions and enzyme activities are presented in Fig. S4. We can observe only smaller HMGCGR activity in comparison with its expression. In the second model correction (model 2), activities of all cholesterogenic enzymes were optimized in a similar way as for HMGCGR. First the optimal solution with no limitation to enzyme activity was found and in the next step the optimal solution was found where the maximal relative gene activity was limited with the relative gene expression of the corresponding genes which is physiologically more acceptable. The results are shown in Figs S5–S8. The results show that it is possible to fit the relative metabolite levels when no limits are set on the enzyme activities; however, substantial improvement of the criterion function with respect to the model where no enzyme activities were adapted was still achieved even with the limited adaptation where the relative enzyme activities were limited below the relative gene expression. The solution is not acceptable in terms of measurement error of the metabolites. For the third modification (model 3), we again assumed total correlation of the enzyme level with changes of the corresponding mRNA levels changes for the late part of cholesterol synthesis, while the measurement errors for metabolites and activity of enzyme E3 is over 8-fold higher for Creml−/− than WT, which is rather unlikely in a real system. Therefore, the seventh and final model modification (model 7 – final model) was introduced to correct for this anomaly, adding the fourth enzyme E5 to eliminate T-MAS form the synthetic pathway on top of previously described novel elimination paths (E3, E4 and E6). Adaptation schemes with limited values of enzyme activities were applied. These results are presented in Figs S17 and S18. The latter model (model seven) showed an ideal fit of the measured and simulated levels of metabolites (Table 1), and enzyme optimization results are shown in Fig. 4. The following simulation experiments were designed to: (a) determine whether all elimination enzymes (E3–E6) are necessary for adequacy of the model that gave us the best fit, and b) validate the possibility that a single enzyme (E3–E6 combined into one) may be involved in the process of elimination; however, we were not able to fit the metabolite levels with the model. Finally, we performed sensitivity analysis on model seven, where the optimal metabolite levels were evaluated with respect to the enzyme levels. The levels of enzymes that were subject to optimization were simultaneously varied within 5% of the optimal values. A random population of 10,000 enzyme levels, normally distributed around their optimal values, was generated and simulated. The resulting population of 10,000 corresponding metabolite levels also showed normal distributions, with standard deviations between 4 and 7% of the optimal levels. The distribution of the criterion value $J$ for the experiment was skewed with a median value of 0.13, steeply descending towards 0, suggesting a relatively narrow minimum of the criterion function and, consecutively, a narrow range of optimal enzyme levels indicative of a well-defined optimization problem. Only the model with elimination paths of DHL (E3), lathosterol (E6), FF-MAS (E4), and T-MAS (E5) can describe the measured metabolite ratios between Creml−/− and WT with sufficient precision and with physiologically acceptable differences between simulated enzyme activities and measured gene expressions.

**Sterols from cholesterol synthesis are metabolized by a variety of CYP enzymes.** Post-lanosterol intermediates from the Bloch and the K–R pathways were tested as substrates with four human CYP enzymes that are known to oxidize cholesterol. A summary of all products identified by comparison with authentic standards,
Figure 3. The cholesterol synthesis pathway, combined with a mathematical model. The underlined genes (gray) and sterol intermediates (black) were measured in the present study. Enzyme (gene) abbreviations are according to Unigene (see Abbreviations in Supplementary information). E1 and E2 present multiple enzymes combined, while E3, E4, E5 and E6 present minimal requirements for mathematical model illustration of the sterol intermediate levels measured. Numbers (%) show initial division of metabolic flux at branching points, while numbers in squared brackets show final model division values. DHL, 24,25-dihydrolanosterol; FF-MAS, follicular-fluid meiosis-activating sterol; T-MAS, testis meiosis-activating sterol; 7-DHC, 7-dehydrocholesterol.
by gas chromatography-mass spectrometry (GC-MS) fragmentation or by NMR, is given in Table 2. Of eleven sterols used as substrates, we were able to definitively identify products from five (7-DHC, zymostenol, lathosterol, desmosterol, and cholesterol). In the remaining cases, the levels of products were either too low to be practical for characterization or identification was not successful, so we provide only the percentages of substrate conversion (Table 3).

Surprisingly, most post-lanosterol intermediates are substrates for cytochrome P450, family 7, subfamily A, polypeptide 1 (CYP7A1), CYP27A1, and cytochrome P450, family 46, subfamily A, polypeptide 1 (CYP46A1) in vitro. The side chain cleavage enzyme CYP11A1 is more specific: only 7-dehydrocholesterol and desmosterol, the immediate precursors of cholesterol, were substrates for this enzyme (Table 3).

CYP7A1, CYP11A1, CYP27A1, and CYP46A1 converted desmosterol to 7α-hydroxydesmosterol, pregnenolone, 27-hydroxydesmosterol, and 24S,25-epoxycholesterol and 27-hydroxydesmosterol, respectively. Incubation of CYP7A1 with desmosterol yielded one product in the liquid chromatography-mass spectrometry (LC-MS) profile (Fig. 5.1). In the 1H NMR spectrum one new peak was present (compared to the substrate

| Gene     | Change from WT Mean (95% CI) | Sterol measured Mean (95% CI) | simulated |
|----------|------------------------------|-------------------------------|-----------|
| Hmgcr    | 1.65 (1.38–1.91)             |                               |           |
| Fdft1    | 3.09 (2.48–3.71)             |                               |           |
| Sqle     | 1.42 (1.06–1.77)             |                               |           |
| Lss      | 1.14 (0.65–1.63)*            |                               |           |
| Cyp51    | 0.39 (0.17–0.68)             | Lanosterol 3.28 (2.59–4.16)   | 3.28      |
| Dhc24    | 1.21 (0.94–1.48)*            |                               |           |
| E3       |                              |                               |           |
| E4       |                              |                               |           |
| Tm7sf2   | 0.87 (0.55–1.18)*            |                               |           |
| E5       |                              |                               |           |
| Sc4mol   | 1.30 (0.61–1.99)*            | T-MAS 0.26 (0.19–0.36)        | 0.26      |
| Nsdhl    | 0.87 (0.50–1.24)*            |                               |           |
| Hud17b7  | 1.55 (1.06–1.34)             |                               |           |
| Elp      | 1.81 (1.47–2.15)             | Lathosterol 0.53 (0.46–0.61)  | 0.53      |
| E6       |                              |                               |           |
| Sc5d     | 0.90 (0.70–1.11)*            |                               |           |
| Dhc7     | 0.29 (0.05–0.54)             | 7-DHL 0.86 (0.78–0.94)        | 0.86      |
| Cyp11a1  | 1.74 (1.28–2.21)             | Desmosterol 0.88 (0.78–0.99)  | 0.88      |
|          |                              | Cholesterol 1.15 (1.08–1.23)  | 1.15      |

Table 1. Experimental and simulated results. Experimental results (with corresponding 95% confidence intervals, real measurements) in comparison with simulated data (sim) for expression of several genes and cholesterol synthetic sterol intermediates of the best fit model (Experiment Seven). Values represent the ratio of Crem−/− versus WT. *Statistically non-significant; CI, confidence interval.
desmosterol) at δ 3.86 ppm with integration to one proton, indicative of a –CH group (Fig. 6.1), confirmed by heteronuclear single-quantum correlation (NMR) spectroscopy (HSQC) (Fig. S19). Correlation (NMR) spectroscopy (COSY) showed that the protons at δ 3.86 and 5.6 ppm are coupled to each other, indicating that these protons are on adjacent carbons (Fig. 6, Figs S19 and S20). The product was identified from 1H NMR (Fig. 6, Figs S19 and S20) and 13C NMR (not shown) as 7α-hydroxydesmosterol, based on chemical shifts of the H7 and H6 protons.

Incubation of CYP11A1 with desmosterol or 7-dehydrocholesterol yielded one product in the LC-MS profile in each case. CYP11A1 cleaves the side chain of cholesterol in a three-step reaction, resulting in pregnenolone. The same product is expected starting from desmosterol. The product (t_R 1.43 min, Fig. 5.2) was identified as pregnenolone by comparison with an authentic standard.

Incubation of CYP27A1 with lathosterol, 7-DHC, or zymostenol yielded two products in the LC-MS profile in each case (results not shown), while desmosterol yielded only a single product (Fig. 5.3). The minor product from lathosterol was identified as 25-hydroxylathosterol using GC-MS (Fig. 6.2). GC-MS fragmentation of a TMS ether showed a major peak at m/z 131, characteristic of a 25-hydroxy product (vide infra).

Similarly, fragmentation analysis of the products formed from 7-dehydrocholesterol and zymostenol yielded 25-hydroxy-7-dehydrocholesterol and 25-hydroxyzymostenol, respectively. The major product formed from lathosterol was identified as 27-hydroxylathosterol by NMR (Fig. 6.3). The two protons attached at C27 (H27) appear to be split, although they are attached to the same carbon atom (C27), because of their diastereotopic nature. In the 1H NMR spectrum (compared with the lathosterol substrate) there were two new peaks at δ 3.42 and 3.50 ppm, with integration to one proton each (Fig. 6.3), indicative of either two –CH or one –CH2 group(s). HSQC NMR confirmed that these protons are attached to a methylene group (CH 2) and were also split in that spectrum (Fig. S21). The observed splitting patterns in both 1H and HSQC NMR matched with the spectra of standard 27-hydroxycholesterol, confirming the product as 27-hydroxydesmosterol.

Table 2. Summary of enzymatic assays with CYPs and various sterols. Reactions with cholesterol served as positive control.

| CYP    | Sterols         | Products                      | Ref.    |
|--------|-----------------|-------------------------------|---------|
| CYP7A1 | Zymostenol      | n.d.                          | This work |
|        | Lathosterol     | 7-Ketocholestanol             | 36      |
|        | 7-DHC           | Cholestanol-7α,8α-epoxide     |         |
|        | Desmosterol     | 7α-Hydroxydesmosterol         |         |
|        | Cholesterol     | 7α-Hydroxycholesterol         |         |
| CYP11A1| Zymostenol      | n.r.                          |         |
|        | Lathosterol     | n.r.                          |         |
|        | 7-DHC           | 7-Dehydropregnenolone         |         |
|        | Desmosterol     | Pregnenolone                  |         |
|        | Cholesterol     | Pregnenolone                  |         |
| CYP27A1| Zymostenol      | 25-Hydroxyzymostenol          | This work |
|        | Lathosterol     | 25-Hydroxyzymostenol          |         |
|        | 7-DHC           | 25-Hydroxy-7-dehydrocholesterol |         |
|        | Desmosterol     | 25-Hydroxydesmosterol         |         |
|        | Cholesterol     | 25-Hydroxycholesterol         |         |
| CYP46A1| Zymostenol      | 24-Hydroxyzymostenol          | 20      |
|        | Lathosterol     | 24-Hydroxyzymostenol          |         |
|        | 7-DHC           | 24-Hydroxy-7-dehydrocholesterol |         |
|        | Desmosterol     | 245,25-Epoxycholesterol       |         |
|        | Cholesterol     | 245-Hydroxycholesterol        |         |

* n.d. could not determine (reaction detected, insufficient separation)

* n.r. no reaction detected

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Similarly, fragmentation analysis of the products formed from 7-dehydrocholesterol and zymostenol yielded 25-hydroxy-7-dehydrocholesterol and 25-hydroxyzymostenol, respectively. The major product formed from lathosterol was identified as 27-hydroxyzyzostenol by NMR (Fig. 6.3). The two protons attached at C27 (H27) appear to be split, although they are attached to the same carbon atom (C27), because of their diastereotopic nature. In the 1H NMR spectrum (compared with the lathosterol substrate) there were two new peaks at δ 3.42 and 3.50 ppm, with integration to one proton each (Fig. 6.3), indicative of either two –CH or one –CH2 group(s). HSQC NMR confirmed that these protons are attached to a methylene group (CH 2) and were also split in that spectrum (Fig. S21). The observed splitting patterns in both 1H and HSQC NMR matched with the spectra of standard 27-hydroxycholesterol, confirming the product as 27-hydroxydesmosterol.
with that obtained from oxidation of desmosterol by CYP46A1 (t_R = 3.94 min, Fig. 5.4), which was identified as 27-hydroxydesmosterol in our earlier work20.

Table 3. Metabolism of sterol intermediates from the Bloch and Kandutch-Russell (K-R) pathway by CYP enzymes that are known to metabolize cholesterol. For percent conversion, each CYP was present (in a reconstituted system) at 1 μM and the substrate concentration was 10–25 μM. The reaction time was 15 min. Shown is the % conversion of substrates to products that were not identified. The identified products (indicated with a “+” sign) are presented in Table 2. The k_cat for formation of pregnenolone was 4.6 ± 0.15 min⁻¹, K_m 1.2 ± 0.36 μM (this work). The k_cat for formation of 24S,25-epoxycholesterol was 0.033 ± 0.001 min⁻¹, K_m 2.17 ± 0.28 μM29. The k_cat for formation of 27-hydroxydesmosterol was ~0.044 min⁻¹20. The k_cat for formation of 7-ketocholesterol was 2.2 ± 0.1 min⁻¹, K_m 1.1 ± 0.1 μM36. The k_cat for formation of 24-hydroxy-7-dehydrocholesterol was 0.024 ± 0.001 min⁻¹, K_m 0.24 ± 0.008 μM and the k_cat for formation of 25-hydroxy-7-dehydrocholesterol was ~0.11 min⁻¹20.

Figure 5. LC-MS profiles of desmosterol incubation with different CYP enzymes. (1) CYP7A1, (2) CYP11A1, (3) CYP27A1, and (4) CYP46A1. An APCI⁺ ionization mode was used, and m/z 383 (for hydroxy and epoxy products, with loss of H₂O to give m/z 383 [MH-18]+) was monitored in each case except CYP11A1 where it was monitored at m/z 299 (for pregnenolone, with loss of H₂O to give m/z 299 [MH-18]+). A, assay without enzyme. B, assay with enzyme.
Incubation of CYP46A1 with lathosterol, 7-dehydrocholesterol, zymostenol (data not shown), or desmosterol (Fig. 5.4) yielded two products in the LC-MS profile. The two products from lathosterol were identified as 24-hydroxy- and 25-hydroxylathosterol using GC-MS (Fig. 6). GC-MS fragmentation showed major peaks at \[ m/z = 145 \] and 503 for the 24-hydroxy product and at \[ m/z = 131 \] for the 25-hydroxy product (Fig. 6.4), characteristic of 24-hydroxy and 25-hydroxy products. These fragmentation patterns matched those of standard 24- and 25-hydroxycholesterol (data not shown). Similarly, fragmentation of the TMS derivatives of products led to the characterization of 24- and 25-hydroxy-7-dehydrocholesterol formed from 7-dehydrocholesterol and 24- and 25-hydroxyzymostenol as products of zymostenol. The two products from desmosterol were identified as 27-hydroxydesmosterol and 24\(^S\),25-epoxycholesterol in our earlier work\(^{20}\).

**Discussion**

Cholesterol is an important constituent of mammalian cell membranes. It is also the precursor of various steroidal hormones, e.g. cortisol, aldosterone, progesterone, androgens, and estrogens. About one-fourth of cholesterol arises from dietary intake and about three-fourths from endogenous synthesis\(^ {21}\). Therefore, the cholesterol synthesis pathway is of considerable biological importance in reproductive organs and is orchestrated by the cAMP-dependent signaling pathway in mouse testes\(^ {22,23}\).

We previously observed that the negative cholesterol feedback regulation mediated by transcription factors of the sterol regulatory element binding transcription factor (SREBF) family is not sufficient to explain the behaviour of the cholesterol synthesis network under various physiological conditions. We proposed that the modulation of post-lanosterol cholesterol synthesis requires interactions between cAMP signaling and cholesterol feedback regulation in both testes\(^ {15}\) and in somatic cells\(^ {24}\). In the present study, we propose for the first time that the role of cAMP signaling might be to activate the ‘branching’ metabolism of cholesterol synthesis intermediates when the synthesis pathway is not coupled. This assumption was deduced from mRNA and sterol metabolite measurements in \( Crem^{-/-} \) mice compared to wild-type, coupled with modeling of the post-squalene cholesterol synthesis pathway\(^ {25}\), which is distinct from modeling cholesterol synthesis focused on HMGCR as the major regulatory point\(^ {26}\). Our model proposed branches from the main cholesterol synthesis pathway, described by virtual...
enzymes E3–E6. The inclusion of CYP11A1 and the virtual enzymes (E3–E6) to the model shows an ideal fit with the measured metabolites (Table 1), where optimization of enzyme levels was based on measured mRNA levels of cholesterogenic genes. Model optimization of HMGCR showed a decrease to 0.5-fold in enzyme expression in Cre<sup>−/−</sup> compared to WT animals, while the experimental mRNA level was increased 1.65-fold. This result can be explained on the basis of previous <i>in vitro</i> and <i>in vivo</i> studies showing that lanosterol, the first sterol intermediate in cholesterol synthesis, potently stimulates ubiquitination and consequent degradation of HMGCR, whereas cholesterol has no effect<sup>27</sup>. Due to the diminished activity of the lanosterol 14α-demethylase in Cre<sup>−/−</sup> mice<sup>15</sup>, lanosterol accumulates in the testes and can lead to an increase in HMGCR degradation. Furthermore, the lanosterol product 27-hydroxylanosterol is even more potent in accelerating HMGCR degradation<sup>27</sup>.

We hypothesize that enzymes E3–E6 are CYP enzymes that metabolize cholesterol and some other sterols, representing new biologically relevant branches from the late portion of cholesterol synthesis. An established branch point is the side chain cleavage enzyme CYP11A1, best known for its initial step in catalysis of steroidogenesis in adrenals and gonads through cAMP-mediated signals<sup>28–30</sup>. In addition, CYP11A1 is important also in vitamin D metabolism and can metabolize 7-dehydrocholesterol to 7-dehydroprogrenenolone<sup>1</sup>. Our measurements showed significantly higher expression of Cyp11a1 in testes of Cre<sup>−/−</sup> mice compared to controls, and the branching reaction of CYP11A1 from 7-DHC was accordingly added to the model. The enzyme activity assays show that desmosterol can also be converted to pregnenolone by CYP11A1 as another branch from the cholesterol pathway towards steroid hormones. Even though this reaction has been proposed previously<sup>31</sup>, evidence that this exists has been presented only during this past year by us (in this paper) and others<sup>32</sup>. Based on our data, CYP11A1 removes the sterol side chain only from 7-dehydrocholesterol, desmosterol, and the typical substrate cholesterol (Tables 2 and 3), in contrast with molecular modeling predictions about a much broader substrate specificity<sup>32</sup>

The second branching candidate enzyme is CYP46A1, initially thought to be expressed mainly in the brain. Recent entries in the Human Protein Atlas show that it is expressed in 41 of 80 tissue cell types analyzed, including brain and testis (http://www.proteinatlas.org/). Interestingly, in the mouse testis the Cyp46a1 mRNA level was below the detection level. CYP46A1 has a potentially broad substrate specificity for ring-modified sterols. The reported natural substrates (other than cholesterol) are 7-DHC<sup>20,33</sup> and desmosterol<sup>20</sup>. We show that the majority of sterol intermediates in cholesterol synthesis are substrates for CYP46A1, with the exceptions of lanosterol and DHL (Tables 2 and 3).

CYP27A1 is the next proposed branching enzyme. Initially it was characterized in terms of roles in bile acid synthesis, but later a much broader substrate specificity was found<sup>34</sup>. We showed that all sterols tested are oxidized by CYP27A1 (Table 3), being converted to 25- and 27-hydroxy derivatives, when product identification was possible (Table 2). Thus, CYP27A1 is a “general” sterol metabolizing enzyme, but we have not addressed the question of whether all 27-hydroxylated sterols can be metabolized to bile acids.

The last enzyme tested, CYP7A1, is not relevant for the testis but can represent a new branching enzyme in the liver, where it is expressed rather exclusively. It was shown previously that CYP7A1 is active towards desmosterol<sup>25</sup>, lathosterol, and 7-DHC<sup>26</sup>. Similarly, as shown for CYP46A1, the majority of sterols involved in cholesterol synthesis are substrates also for CYP7A1, with the exception of lanosterol and DHL (Tables 2 and 3).

Due to the recent discoveries, we questioned what the relationship is between sterols downstream of lanosterol, resulting from the herein described CYP metabolism, and the ones identified as potential RAR-related orphan receptor gamma (OR<i>γ</i>) ligands<sup>37</sup>. The general structure of endogenous ROR<i>γ</i> ligands<sup>37</sup> contains a 3β-hydroxy or a carbonyl moiety at C3, a possible methyl group at C4 (preferably in β conformation) or a carbonyl group preferred at C4α, and double bonds at either C5 or C8 of the sterol ring and C25 (26) of the side chain (Fig. 7). We have shown that CYP27A1 oxidizes most sterols from the Bloch and the K-R pathways (Table 3) by attaching a hydroxyl group at C25 or C27 of the sterol side-chain. Compared to cholesterol, 25-hydroxycholesterol and 7α,27-hydroxycholesterol are potent ROR<i>γ</i> activators at low concentrations<sup>37</sup>. Also CYP7A1 metabolites (7α-hydroxysterols) and CYP46A1 metabolites (addition of a hydroxyl group to C24 or C25 or an epoxy group) could activate ROR<i>γ</i><sup>37</sup>. While the substrate specificity of CYP enzymes differs for the sterol intermediates (and is generally lower compared to their canonical substrates), the flux through the cholesterol pathway can change dramatically under pathological conditions. For example, in <i>Cyp51</i> liver conditional knockout mice with pathology similar to non-alcoholic hepatitis, the CYP51 substrates lanosterol and DHL accumulate while sterols downstream the pathway, such as zymosterol and other potential ROR<i>γ</i> natural ligands, are diminished<sup>39</sup>. Increased concentrations of lanosterol and its metabolites might, in part, be responsible for reconstitution of ROR<i>γ</i> activity that remained in <i>Cyp51</i>−/− fibroblasts despite a complete <i>Cyp51</i> ablation<sup>37,40</sup>.

Conclusions

Mathematical modeling of cholesterol synthesis led to the proposal of virtual enzymes branching from the post-lanosterol pathway to explain the experimental data. Activity measurements identified CYP27A1 as the most likely candidate for virtual enzymes E3–E6 in the testis. CYP27A1 metabolized FF-MAS (substrate of E4), T-MAS (substrate of E5), and lathosterol (substrate of E6), probably to 27-hydroxylated sterols. If the same branches are operative in the liver, CYP7A1 might also have a role. The role of CYP46A1 in the testis is not clear at the moment because we could not detect <i>Cyp46a1</i> mRNA expression, but the enzyme might participate in sterol metabolism in other tissues. Dihydrolanosterol (a substrate of E3) was metabolized only by CYP27A1, which is thus the best candidate for E3 in all tissues. Irrespective of the tissue, the early intermediates lanosterol and DHL have less opportunity to escape from the pathway, compared to the intermediates immediately before cholesterol, desmosterol, and 7-DHC, which can be oxidized by CYP11A1, CYP46A1, CYP7A1, and CYP27A1. Sterols from the Bloch and the K-R pathways have the potential to be metabolized further to oxyosters with potentially novel biological activities.
Materials and Methods

In Vivo Animal Experiments. Animals. Forty-two WT and 33 Crem−/− male mice of the mixed strain (129S2/SvPasCrIf in C57BL/6JrJ) were maintained in a temperature and humidity controlled room under a 12:12 h light:dark cycle (light on at 7:00 am, light off at 7:00 pm) with free access to food (Harlan Teklad 2916) and water. The mice were acclimatised (entrained) to this light/dark cycle for at least one month. Crem−/− mice originate from the laboratory of Dr. P. Sassone-Corsi, IGBMC, Strasbourg, and all experiments were performed two years after transfer of animals to Ljubljana. Mice were adults, aged from 56 to 81 days at time of sacrifice. To acquire sufficient information for statistical analysis, identical experimental sampling procedures were repeated for various experimental dates (in April, June, and August). For the two genotype groups, mice were chosen at random over a 24 h sampling procedure.

The experiment was approved by the Veterinary Administration of the Republic of Slovenia (license number 34401-9/2008/4) and was conducted in accordance with the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes (ETS 123), as well as in accordance with National Institutes of Health guidelines for work with laboratory animals.

Tissue samples. The mice were sacrificed by cervical dislocation under dim red light, every 4 h over the 24 h period, starting on the second day after their transfer to dark/dark conditions for 24 h. immediately after they were sacrificed, the testes were excised, snap frozen in liquid nitrogen, and stored at −80 °C for later analysis. Altogether 75 testicular samples from the mixed strain (129S2/SvPasCrIf in C57BL/6JrJ) were used for sterol extraction (for further details see references41,42).

RNA extraction. Total RNA was isolated from frozen pulverized testis from five WT and five Crem−/− animals according to the manufacturer's instructions (SigmaAldrich, St. Louis, MO, United States). cDNA preparation and qPCR were performed as described41. Reference gene selection and normalization of qPCR data was done as described43.

Sterol extraction and GC-MS analysis. The frozen testes (50–100 mg per mouse) were dissolved in 2 ml of Folch solution (chloroform/ methanol; 2:1; v/v) under argon and sealed with a Teflon-lined cap. After 24 h at room temperature, 50 μl of the extract for cholesterol and 400 μl of the extract for the cholesterol precursors was transferred into new vials. The internal standards were added as 2 μg of hexadeutrium-labelled cholesterol for the cholesterol analysis and 150 ng of tetradeutrium-labelled lathosterol for the cholesterol precursors. After hydrolysis of the esterified sterols, 3 ml cyclohexane was added, and the upper (organic) phase was transferred to new glass vials; the extraction was repeated once more with a further 3 ml of cyclohexane. The extracts were pooled and evaporated under a stream of argon in heating blocks at 60 °C in preparation for derivatization. The samples were
analyzed according to a gas chromatography–mass spectrometry (GC–MS) methodology described previously. Each sample was analyzed for six post-squalene sterols: lanosterol, T-MAS, lathosterol, desmosterol, 7-DHC, and cholesterol. The quantities of the total (i.e. free and esterified) sterols of these mouse testis samples were normalized to the wet tissue weight.

Statistical analysis. The R statistical programming language was used for the statistical analysis and the package ggplot2 for the graphical presentations. Comparison of gene expression between the WT and Crem−/− lines was performed with t-tests on the logarithmic data. Sterol intermediates were analyzed by ANOVA, adjusted on two extraneous variables (age and experiment date).

Mathematical Modeling. Cholesterol synthesis network model. A mathematical model of cholesterol synthesis was applied based on object-oriented modeling by combining substance containers (concentrations) and reactions (fluxes) as objects. The basic principles and equations for describing concentrations of substances, reactions that define the fluxes through the metabolic network (enzyme reaction model), and mRNA and enzyme formation and degradation are described in detail in the Supplementary Information. The model was implemented in Dymola 5.3 (Dynasim AB, Lund, Sweden) and simulated with a Petzold integration routine (dassl).

Simulation of cholesterol synthesis pathway in the testis of Crem−/− mice: optimizing the model according to the measured genes and metabolites. The initial cholesterol synthesis network model was composed of the pathways confirmed in KEGG, LIPID MAPS, and BioCyc (see Supplementary Fig. S1). The modeling aim was to mechanistically describe the flux distribution and enzyme activities that are involved in processes triggered by Crem−/−. A highly regulated pathway is capable of adapting to wide variety of disruptions, some as heavy as complete inactivation of certain enzymes in the pathway through genetic disorder, while the underlying mechanisms are still not understood due to their high robustness and redundancy. The simplest possible model design was used, where all model values were normalized. The normalized values enabled direct comparison of Crem−/− vs. WT ratios obtained from the experiments and reduced the number of the free parameters of the model to minimum.

The model parameters were set by defining arbitrary initial metabolic flux through the network, with 75% of the flux arising from de novo cholesterol synthesis and 25% from other resources (dietary cholesterol intake). The initial flux distributions within the network are described in Fig. 3.

Model setup. Michaelis-Menten reaction kinetics were used to describe enzyme reactions (hyperbolic plots, fit using non-linear regression). Constant protein biosynthesis and biodegradation with linear kinetics is used in the model. mRNA is also constantly synthesized and degraded with linear kinetics. The flux of protein synthesis depends on the corresponding mRNA concentration and has also linear character. Feedback on gene expression of cholesterogenic enzymes through SREBF2 was not active in the model and mRNA levels were set to values observed during the experiments. All fluxes in the model were considered at steady state at the beginning of the simulation which resulted in a system of linear equations that yielded the values of model parameters. For each reaction, its initial reversibility (ratio of reverse and forward flux) and relative steady-state initial complex concentration were set and, to conserve flux through the network, for each branching point of the network the ratio of dividing initial fluxes was also set. The influence of the reversibility was negligible so all reactions were set to 0.01, and the influence of the steady-state initial complex concentration strongly effects the length of the transient phenomena after the network disturbance. However, it does not affect the new steady-state of the network and therefore it was set to 0.01 (1% of the initial normalized value of the substrate or enzyme) to shorten the transient phenomena (small values of steady-state complex concentration indicate fast reactions and thus faster transient phenomena). The division of fluxes at branching points can significantly influence the new steady-state of the network therefore they must be carefully chosen. Using literature data for mice testis we were able to set the flux distribution as described in the Supplementary Information.

Simulation setup. Eight hypotheses were tested with the model.

1. The textbook model is sufficient to describe the Crem−/− situation in comparison with the WT situation and the only influence on enzyme activity is through gene expression modulation.
2. The textbook model correctly describes the Crem−/− - WT relations, enzyme activity is regulated only through gene expression except for HMGCR where known degradation of HMGCR through high levels of lanosterol can produce lower activity than expected by just observing Hinger expression.
3. The textbook model correctly describes the Crem−/− - WT relationship; however, the enzyme activities are not solely regulated by gene expression but may be affected by other factors.
4. The textbook model is too simple to describe the Crem−/− - WT relations and DHL is eliminated from the pathway via an alternative route. Enzyme activities are regulated through gene expression as well as protein degradation mechanisms.
5. The textbook model is too simple to describe the Crem−/− - WT relations, and DHL and lathosterol are eliminated from the pathway via alternative routes. Enzyme activities are regulated through gene expression as well as protein degradation mechanisms.
6. The textbook model is too simple to describe the Crem−/− - WT relations and DHL, lathosterol, and FF-MAS are eliminated from the pathway via alternative routes. Enzyme activities are regulated through gene expression as well as protein degradation mechanisms.
Optimal enzyme activities and flux distribution ratios were estimated using criterion minimization. The criterion was the sum of squared differences between measured and simulated relative changes in steady-state concentrations of metabolites: lanosterol, T-MAS, lathosterol, desmosterol, and cholesterol. The initial steady-state for all model concentrations was 1; therefore, the steady-state values after perturbation were already relative changes of concentrations. Simulation end time was expected well beyond transient phenomena and was monitored throughout the optimization process through concentration derivative at the simulation end. Nelder-Mead optimization routine was used. Multiple local minima were expected since in most case the number of parameters to be optimized was higher than the number of metabolite concentrations to be fitted. This creates a situation where degrees of freedom are usually higher than necessary for solving the problem which results in several combinations that produce the same result. To explore the multiple solutions problem, the optimization was started from several starting points. One initial point was original enzyme activity, the next initial point was original enzyme activity except for HMGCR where reduced activity was used, and the final initial point was optimal estimated enzyme activity of the final model. The enzyme activity was modulated by tuning the enzyme elimination coefficient.

**Enzyme Activity Experiments. Codon optimization and construction of expression vectors.** On-line software (Integrated DNA Technologies, Coralville, IA; http://www.idtdna.com/CodonOpt) was used for codon optimization for human CYP11A1. A cDNA containing an optimized coding sequence (Fig. S22) and a C-terminal (His)_6 tag was synthesized and ligated into a pcW expression vector (using Ndel and HindIII restriction sites) by Genewiz (South Plainfield, NJ, United States).

A tricistronic plasmid construct containing human CYP27A1, adrenodoxin (ADX), and adrenodoxin reductase (ADR) was used as a template for PCR to amplify the cDNA of CYP27A1. The 5’ primer for CYP27A1 insertion into the pcW was designed to introduce the Ndel restriction site within the initiation codon ATG (Met). The sequence of the 5’ primer was CATATGGCTCTTCCATCTGATAA. The 3’ primer was template-specific but with a C-terminal (His)_6-tag, stop codon (TAA), and XbaI restriction site. The sequence of the 3’ primer was GCAGTTCCCTGAGAGACAGTGCAACATCACCACCATACATCTAGA. The PCR products were digested and ligated into the pcW vector, and the sequence of the insertion was confirmed by nucleotide sequence analysis.

**Enzyme expression, purification, and activity assays.** Expression and purification of CYP7A1 and CYP46A1 were described earlier. Recombinant rat NADPH-cytochrome P450 reductase (CPR) was expressed in *Escherichia coli* and purified as described.

Detailed protocols for expression and purification of CYP7A1 and CYP27A1 with ferrous-carbon monoxide vs. ferrous difference spectra (Fig. S23) as well as ADX and ADR are provided in the Supplementary Information.

Activity assays were carried out in a 0.5 ml reaction volume containing the indicated sterol (7-DHC, cholesterol, lanosterol, DHC, pregnenolone, 7-dehydrodesmosterol, and lathosterol), the CYP enzyme and its redox partner: CPR for CYP46A1 and CYP7A1 or ADX and ADR for CYP27A1. The enzyme activity was modulated by tuning the enzyme elimination coefficient.
Y. Xiao and L. D. Nagy for preparation of ADX and ADR. (Karolinska Institutet, Stockholm) for providing advice and help with the GC-MS analysis of sterol levels and

This study was supported by the Slovene Research Agency (core funding program P1-0104) and US National Institutes of Health grant R37 CA090426. J. Acimovic is indebted to the Slovene Human Resources Development and Scholarship Fund for a fellowship grant (Ad-futura programme). The authors thank Prof. I. Björkhem (Karolinska Institutet, Stockholm) for providing advice and help with the GC-MS analysis of sterol levels and Y. Xiao and L. D. Nagy for preparation of ADX and ADR.

**Acknowledgements**

This study was supported by the Slovene Research Agency (core funding program P1-0104) and US National Institutes of Health grant R37 CA090426. J. Acimovic is indebted to the Slovene Human Resources Development and Scholarship Fund for a fellowship grant (Ad-futura programme). The authors thank Prof. I. Björkhem (Karolinska Institutet, Stockholm) for providing advice and help with the GC-MS analysis of sterol levels and Y. Xiao and L. D. Nagy for preparation of ADX and ADR.
Author Contributions
J.A. carried out sterol intermediate extractions, GC-MS and data analyses, participated in mathematical modeling, and wrote the manuscript. S.G. prepared the human CYP enzymes and performed enzymatic assays and analyses of the sterol products. R.K. carried out qPCR analysis. M.G. participated in the planning, sampling, and data analyses and provided useful discussions. M.P. is responsible for animal experiments and participated in sampling, design of the study and provided useful discussions. A.B. constructed the mathematical model and performed simulation studies, participated in data analysis, and provided useful discussion. Z.U. performed the RORα structure activity relationship. D.R. supervised the study and participated in study design, coordination, and manuscript writing. F.P.G. was involved in the characterization of the in vitro sterol products and wrote part of the manuscript. All authors read and approved the final manuscript.

Additional Information
Supplementary information accompanies this paper at http://www.nature.com/srep

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Ačimovič, J. et al. Cytochrome P450 metabolism of the post-lanosterol intermediates explains enigmas of cholesterol synthesis. Sci. Rep. 6, 28462; doi: 10.1038/srep28462 (2016).

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