

Biochemical Characterisation and Whole Genome Expression Profiling of Cultured Skin Fibroblasts from Two South African Adults with Urinary 3-Hydroxyisovaleric Acid and 3-Methylcrotonylglycine

Zandberg L¹, Erasmus E¹, Suormala T^{2,3}, Mienie LJ¹, Mels CMC⁴ and Van Dijk AA¹

- 1 Biochemistry, Focus Area Human Metabolomics, North-West University, Potchefstroom, South Africa
- 2 Division of Metabolism, University Children's Hospital, Zurich, Switzerland
- 3 Metabolic Unit, University Children's Hospital, Basel, Switzerland
- 4 Hypertension in Africa Research Team, North-West University, Potchefstroom, South Africa

Abstract

We report on the first case of marginal 3-methylcrotonyl-CoA carboxylase (MCC) deficiency in South Africa. Urinary 3-hydroxyisovaleric acid and 3-methylcrotonylglycine were detected in four males of a non-consanguineous family. Only the index patient (NWU001) had non-specific symptoms, the others were asymptomatic. The inherited metabolite profile and partially reduced MCC activity were indicative of marginal MCC deficiency. *In vivo* L-leucine loading confirmed a reduced flux through the leucine degradation pathway. No known deleterious mutations were detected in the open reading frames of *MCCC1* and *MCCC2*. NWU001 was heterozygous for a SNP in *MCCC1* (rs2270968; c.1391A>C, p.H464P). NWU002 was heterozygous for a *MCCC2* splice variant which skips exon 7 and causes an in frame deletion of 38 amino acids that is identical to a predicted shorter *MCCC2* isoform-2 (Q9HCC0-2). Whole genome expression profiles from cultured skin fibroblasts of NWU001 and NWU002 and two healthy adults using Affymetrix® HuExST1.0 arrays detected 14237 significantly differentially expressed transcript IDs of which only 1277 have known annotation and gene association. The underlying molecular interactions, secondary signalling responses and functional relationships of these 1277 transcripts were inspected following a knowledge-based functional analyses approach using Ingenuity Pathway Analysis software. The transcriptome had a footprint of oxidative stress, disruption of energy homeostasis, inflammation, impaired cellular maintenance and repair mechanisms. Of note was the significant up regulation of the fatty acid amide hydrolase variant 2 (*FAAH2*) HuChrX transcript in the anandamide degradation canonical pathway. The observations that the two MCC transcripts were not significantly differentially expressed and that more than 90% of the significantly differently expressed transcripts are still poorly annotated further support the notion that secondary factors other than the MCC loci impact on the MCC deficiency phenotype.

Keywords: 3-methylcrotonyl-CoA carboxylase deficiency; Oxidative stress; Affymetrix® HuExST1.0 array; 3-Hydroxyisovaleric acid; 3-Methylcrotonylglycine; Leucine catabolism

Corresponding author: Van Dijk AA

✉ albie.vandijk@nwu.ac.za

Biochemistry Division, North-West University, Private Bag X6001 Noordbrug, 2520 Potchefstroom, South Africa.

Tel: +27 18 299 2317

Fax: +27 18 299 2316

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Introduction

There is an increasing awareness that although inborn errors of metabolism are considered simple monogenic disorders caused by a deficiency of a single enzyme, they often act as complex

diseases [1-5]. This is evident from the often broad spectrum of clinical symptoms and highly variable time of onset for the same disease [4,6-10]. One such disease is isolated 3-methylcrotonyl-CoA carboxylase (MCC) deficiency (MIM #210200 and MIM

#210210) [11-14]. MCC deficiency is an autosomal recessive disorder of the leucine catabolism which usually presents with elevated levels of urinary 3-hydroxyisovaleric acid (HIVA) and 3-methylcrotonylglycine (MCG) [15-16]. Newborn screening (NBS) revealed that MCC deficiency is the most frequently detected organic aciduria [13,17,18]. MCC deficiency is regarded as a disease with a low penetrance [19] since less than 10% of individuals with a metabolite profile indicative of MCC deficiency develops [14]. The clinical, biochemical, and enzymatic characterization of MCC deficiency have been well documented, but the pathomechanism remains largely unknown [9,15,20-26].

MCC is one of four biotin-dependent carboxylases and is comprised of two non-identical subunits, alpha (MCCA) and beta (MCCB), which forms a $\alpha_6\beta_6$ hetero-dodecamer [27-29]. The two subunits are encoded by nuclear genes and have signal peptides that target them to the mitochondrion [30]. The alpha subunit is encoded by the *MCCC1* (formerly called *MCCA*) gene located on chromosome 3q25-q27 and has 19 coding exons. The beta subunit is encoded by the *MCCC2* (formerly called *MCCB*) gene located on chromosome 5q12-q13 and has 17 coding exons [31-33]. About 132 disease-causing mutations have been reported for the *MCCC1* and *MCCC2* genes [19] but no prevalent mutation has been identified and there is no clear genotype-phenotype correlation [17,21,31]. The MCC activity of patients with deleterious homozygous or compound heterozygous mutations is usually reduced to <5% of the mean activity of the controls [15,21,22,31], but a few cases have been reported with residual MCC activity, up to 34% [19,22,26] and some individuals with urinary metabolites indicative of MCC deficiency who only have a single heterozygous MCC mutation have almost normal MCC activity [17]. There is an increasing awareness of the difficulty to diagnose MCC deficiency because some patients present with an atypical metabolic profile with only trace amounts or even no detectable urinary MCG [22], the variability and non-specific nature of its symptoms and the growing number of individuals presenting with MCC deficiency associated metabolites but with only a single mutation in *MCCC1* or *MCCC2* [17]. Recently, analyses of the first exome sequence data from MCC deficiency cases [34] revealed that individuals who have the same mutation most likely share a common ancestor, that rare mutations outside of MCC genes can cause non-specific phenotypes hitherto attributed to MCC deficiency in genetically closely related patients and that the clinical consequences of MCC deficiency could be less pervasive than generally accepted. The authors also stated that further studies should be done to assess the value of treating asymptomatic individuals with MCC deficiency such as those who are identified through NBS programmes [34].

MCC deficiency has not yet been reported in South Africa. In this paper we present results from a non-consanguineous South African family of which four males presented with a metabolic profile with borderline high levels of HIVA and detectable amounts of MCG which suggest MCC deficiency. Only one of the affected males experienced non-specific symptoms since early adulthood, whereas the other three remained asymptomatic. Our mutation analyses of the open reading frames (ORF) of *MCCC1* and *MCCC2*,

enzymatic studies and *in vivo* L-leucine loading suggest that these individuals have marginal MCC deficiency. Intriguingly, the whole genome expression profile of cultured skin fibroblasts indicated that both *MCCC1* and *MCCC2* were not significantly differentially expressed in cells of the affected individuals compared to the controls. The gene expression profiling and inheritance pattern in this family seem to suggest that the chromosome-X (HuChrX) might impact on the pathomechanism of this disorder.

Clinical Presentation

The index patient (NWU001), a Caucasian male from a non-consanguineous South African family, was 48 years of age when he presented with mild hepatosplenomegaly. "The metabolic screen revealed elevated levels of urinary 3-hydroxyisovaleric acid and 3-methylcrotonylglycine which is usually indicative of MCC deficiency." He was advised to report for metabolic screening. The patient mentioned a 20 year history of frequently occurring symptoms often in association with episodes of chronic fatigue. He reported reduced tolerance to exercise and frequently experienced acute muscle cramps which subsided with rest. He complained of general weakness, poor tolerance to environmental factors and being prone to hay fever, eczema, mouth ulcers and sinusitis. Although the patient had a body mass index of 27.9 (Obese reference range: >26.9), he appeared healthy and not overweight. His blood chemistry was mostly within normal ranges, with the exception of a slightly elevated alanine aminotransferase activity of 41 U/L (Male age 12 months-60 years reference range: 10-40 U/L) and elevated levels of 784 pmol/L VitB12 (Normal reference range: 148 - 616 pmol/L). His total cholesterol was borderline high, 5.5 mmol/L (Borderline high reference range: 5.18-6.19 mmol/L) and he had low HDL, 0.4 mmol/L (Normal reference range: <1.04 mmol/L), normal LDL, 2.67 mmol/L (Normal reference range: <2.85 mmol/L) but very high levels of triglycerides, 6.73 mmol/L (normal reference range: <5.65 mmol/L) [35]. He presented with normal blood pressure, fasting glucose and haematological parameters.

The second family member, NWU002 (male, age 52), sibling of the index patient NWU001 had no clinical symptoms related to that of his brother. However, he also presented with a similar profile of abnormal urinary organic acids and acylcarnitine urinary metabolites as NWU001. At the time of investigation NWU002 already received medication to control persistent high levels of total cholesterol and his total cholesterol level was 4.9 mmol/L (Reference range: 2.8-5.2 mmol/L).

In addition to the index patient and his brother another thirteen family members over three generations (**Figure 1**), ten females (aged, 83, 80, 56, 53, 33, 7, and twins of 2 years) and three males (aged 31, 6 and 4 years), participated in a voluntarily metabolic screen. From the thirteen family members that were screened another two males in the family, the grandsons of NWU002 (NWU003, male, aged 6 years and NWU004, male, aged 4 years) also presented with a similar urinary organic acid profile as that of NWU001 and NWU002 (**Supplementary Table 1**). However, informed consent to include the grandsons (NWU003 and NWU004) for further testing could not be obtained and they were excluded from all further participation in this study.

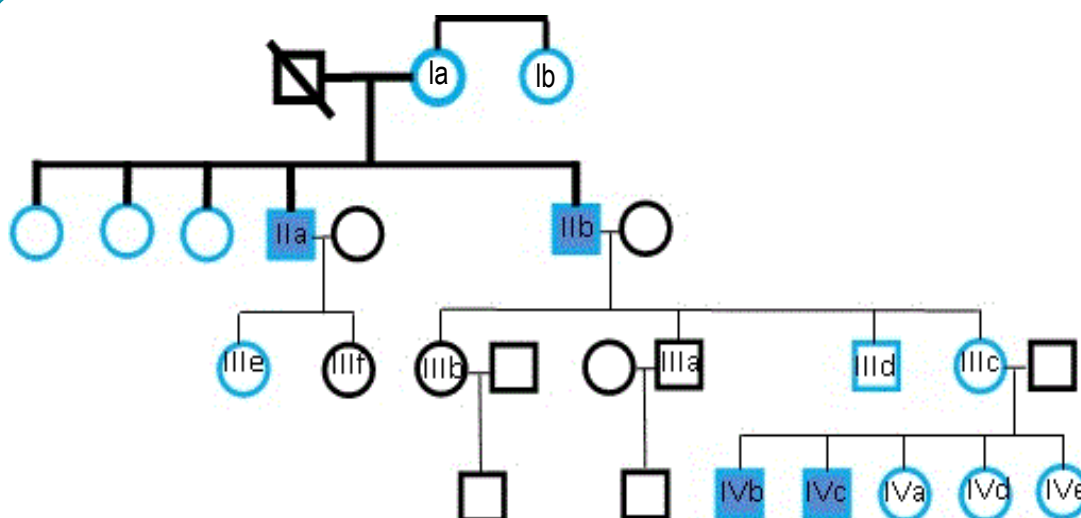


Figure 1 Family tree showing screened and affected family members. The family members that agreed to urinary organic acid and acylcarnitine screening are indicated in blue. The family members indicated in black were not screened. The blue coloured squares represent the index patient NWU001 and the three additional male family members that presented with urinary HIVA and MCG. I: First generation; II: Second generation; III: Third generation; IV: Fourth generation, (a) first person in the generation, (b) second person in the generation, (c) third person in the generation, (d) fourth person in the generation, (e) fifth person in the generation, and (f) sixth person in the generation. NWU001=IIa, NWU002=IIb; NWU003=IVb; and NWU004=IVc.

Methods

Urinary metabolic screening

Creatinine concentration of the urine samples were determined colourimetrically with a modified Jaffe reaction. Urinary organic acids were extracted using liquid-liquid extraction and derivatization as described by Hoffman and Feyh [36]. Trimethylchlorosilane (TMCS) esters of organic acids were analysed on a Hewlett Packard 6890/5973 gas chromatography mass spectrometry (GC-MS) system equipped with a DB-1ms capillary column (30 m × 0.25 mm × 0.25 μm, Agilent Technologies, Palo Alto) as described by Rinaldo [37]. The data was quantified using an automated mass spectral deconvolution and identification system (AMDIS) software. The concentration of identified organic acids were expressed in millimole per mole creatinine, using 4-phenylbutyric acid as internal standard.

Butylated urinary acylcarnitines were analysed according to the method described by Millington [38]. An Agilent 1200 series high pressure liquid chromatography system (Agilent Technologies, Palo Alto), equipped with a 96-well plate autosampler and a tripple quadrupole mass spectrometer was used (G6410, Agilent Technologies, Palo Alto). The system was operated in positive electrospray ionisation (ESI) mode with the mass analyzers set in a precursor of 85 m/z configuration. Acylcarnitines were quantified using deuterated analogues of free carnitine and acylcarnitines as internal standards.

In vivo leucine loading

NWU001, NWU002, and five control individuals (Aged between 24 and 64 years) participated in an L-leucine loading challenge. Each participant received 100 mg L-leucine (Sigma-Aldrich) per kg body weight in orange juice [39]. Urine samples were collected at baseline (T0) and 30 min (T1), 1 h (T2), 3 h (T3), 5 h (T4) and 7 h

(T5) after L-leucine administration. Urinary organic acid and acylcarnitine analyses were conducted on all the samples.

Primary skin fibroblast cultures

Skin biopsies from NWU001 and NWU002 and three unrelated, healthy Caucasian adult control individuals (CON001, male aged 62 years, CON002, female aged 25 years and CON003, male aged 24 years) were obtained from the left inner forearm of each participant by a registered nurse. Primary skin fibroblast cultures were established by GenePath (Centurion, South Africa). Fibroblasts were routinely grown in T25 cm² tissue culture flasks in Dulbecco's Modified Eagle's Medium (DMEM, Lonza), supplemented with 10% foetal calf serum (FCS, Lonza), and 1% Penicillin/Streptomycin (Lonza) antibiotics at 37°C under 5% CO₂. NWU001, NWU002, CON001 and CON002 were also grown in Amniochrome system II[®] medium (Lonza) supplemented with 0,125 mg/ml Penicillin/Streptomycin (Lonza) and 0.256 mg/mL Gentamycin (Lonza) in T25 cm² tissue culture flasks for total RNA isolation and hybridization to Affymetrix Genechip HuExST1.0 arrays. Cells were grown until they reached 80-90% confluence. The cell layers were washed twice with phosphate buffered saline (PBS) and total RNA was isolated directly from the flask.

Enzyme assays

Mitochondrial biotin dependent carboxylase activities were assayed in crude fibroblast lysates by measuring the incorporation of [¹⁴C]-NaHCO₃ into acid-non-volatile products by established methods [40]. For the assay of the level of specific activity and kinetics of MCC, fibroblasts were grown in the routine culture medium supplemented with 10% FCS. This medium is biotin sufficient containing 9 nmol/L biotin, contributed by the natural biotin content of FCS, which is 5-times the mean normal plasma biotin concentration in humans (1.8 nmol/L, range 0.6-4.8, n

= 126) [41,42]. The activity of another mitochondrial biotin dependent carboxylase, propionyl-CoA carboxylase (PCC) that serves as a control enzyme, was determined simultaneously with MCC in the same cell lysate. To determine the apparent K_m values of MCC for the substrates 3-methylcrotonyl-CoA and bicarbonate as well as for ATP, MCC activity was measured with variable concentrations of one of the compounds together with routine saturating assay concentrations of the other components. Thus, the concentration of 3-methylcrotonyl-CoA varied between 0.05 and 3.9 mmol/L (in routine assay 1.5 mmol/L), Na-bicarbonate was between 0.42 and 40 mmol/L (in routine assay 20 mmol/L), and ATP was between 0.04 and 6.3 mmol/L (in routine assay 3.15 mmol/L). All K_m values were derived using Eadie-Hofstee plots. To determine the sensitivity to the activator K^+ , MCC activity was measured with and without 50 mmol/L (optimal concentration) and 100 mmol/L KCl in the assay mixture.

Holocarboxylase synthetase (HCS) activity was estimated indirectly by measuring the sensitivity of fibroblast MCC and PCC activities on biotin depletion of the culture medium [43]. Cells were grown in parallel in a medium with low and high biotin concentration and assayed for MCC and PCC activity. Low-biotin medium was prepared by replacing the FCS of the routine culture medium by newborn calf serum which resulted in the final biotin concentration of 0.1 nmol/L, and high-biotin medium by supplementing the low-biotin medium with 10 μ mol/L biotin. Biotinidase activity was estimated in serum by the colourimetric assay using N-biotinyl-p-aminobenzoate as substrate [44].

Mutation analysis of *MCCC1* and *MCCC2*

Total RNA was extracted using the Qiazol[®] reagent (Qiagen) and the RNeasy[®] total RNA extraction Kit (Qiagen). cDNA was synthesised from total RNA isolated from cultured skin fibroblasts of NWU001 and NWU002 using cloned AMV reverse transcriptase (Invitrogen). Phusion[®] high fidelity DNA polymerase (Finnzymes) was used to amplify *MCCC1* and *MCCC2* from cDNA using published primer sequences [31,33]. All amplification products were purified with the QiaQuick[®] gel extraction kit (Qiagen) and sequenced (Inqaba Biotech). The *MCCC1* and *MCCC2* cDNA sequences were inspected for mutations using FinchTV 1.4.0 (Geospiza, Inc.; Seattle, WA, USA; <http://www.geospiza.com>) and aligned to reference sequence NM_020166 for *MCCC1* and NM_022132 for *MCCC2* using the web based Clustal W (Version 2.1) multiple sequence alignment tool [45].

Whole genome gene expression profiling

HuExST1.0 array preparation and hybridisation: The whole genome expression profile was determined with the Affymetrix[®] GeneChip[®] Human Exon ST 1.0 (HuExST1.0) array for hybridisation (Affymetrix). Total RNA was isolated from fibroblasts of NWU001 and NWU002 and two healthy control adults (CON001 and CON002) using the RNeasy[®] mini kit (Qiagen) with no additional DNase digestion. The yield, quality and integrity of the total RNA preparation was assessed according to the integrity of the 28S rRNA and 18S rRNA, as well as RNA integrity number (RIN) value using a NanoDrop 1000 Spectrophotometer and an Agilent Bioanalyser 2100. Single hybridisations of NWU001, NWU002 and the controls, CON001 and CON002, were prepared. Hybridisation was carried out by the Affymetrix[®] service provider

in South Africa, the Centre of Proteomic and Genomic Research (CPGR), Cape Town. Total RNA from each sample with a RIN >9 was fragmented, labelled, hybridised to HuExST1.0 arrays and scanned according to the Affymetrix[®] protocols and instructions. Output signal intensity data files (.CEL) were generated and assessed to ensure good quality output and analyses.

HuExST1.0 array data analysis and significantly differentially expressed transcript ID-list: The data intensity files (.CEL files) received from CPGR were analysed using Affymetrix[®] Expression Console (Version 1.1, HuEx1-0-st-v2.na32.hg19) and Partek[®] Genomic Suite software (Version 6.1, Copyright, Partek Inc. St. Louis, MO, USA). Pre-processing data quality control analyses were done by using the robust multi-array average (RMA) normalisation method for background correction and using the core-meta probe set as annotation for quality control analyses [46,47]. All data intensity files (.CEL) passed the QC metric and were uploaded with Partek[®] GS software (Version 6.1, Copyright, Partek Inc. St. Louis, MO, USA). The Affymetrix[®] full-meta probe set (HuEx1-0-st-v2.na32.hg19) was applied for annotation. The full-meta probe set included transcript IDs with well-defined annotations as well as predicted transcripts with no known gene associations. A list of differentially expressed, annotated and predicted transcript IDs was generated by means of one way analysis of variance (ANOVA). The significantly differentially expressed transcript list included the transcript IDs that met the filter criteria $P < 0.05$. From the significantly differentially expressed list, a subset of well-annotated transcript IDs associated with transcripts associated with genes of the HuChrX was also compiled.

Functional analyses

Ingenuity Pathway Analysis (IPA) software (Ingenuity[®] Systems, www.ingenuity.com, build version 242990, content version 17199142), was applied to investigate the predicted functional relationships, predicted network associations, biological functions, canonical pathways, regulator-, co-activator- and modifier genes associated with the complete list of annotated differentially expressed transcript IDs. A subset of significantly differentially expressed well-annotated transcript IDs associated with HuChrX was compiled and also analysed with IPA (Ingenuity[®] Systems, www.ingenuity.com, build version 313398M, content version 18841524) to further investigate the possible impact of the HuChrX transcripts on the functional relationships, network associations, biological functions, canonical pathways and regulatory networks of this marginal MCC deficiency. The analysis settings for IPA for both input lists were set to include only well annotated transcript IDs and endogenous chemicals identified in the predefined IPA database with a predicted direct and or indirect relationship in *Homo sapiens*.

Quantitative real-time PCR validation

Independent quantitative real-time PCR (qPCR) analysis was performed to confirm the gene expression results of selected transcripts obtained from the HuExST1.0 array hybridisations. The same total RNA samples were used for both the array hybridisation and qPCR analyses. An amount of 1 μ g total RNA of each sample was reverse transcribed using Cloned-AMV reverse transcriptase (Invitrogen). The cDNA samples were diluted and 100 ng was used in each of the validation reactions which were

prepared in triplicate. TaqMan[®] hydrolysis probe chemistry based gene expression assays for *ACOX2* (Hs00185873_m1), *AWAT2* (Hs01040631_m1), *HADHA* (Hs00426191_m1), *HMGCL* (Hs00609311_m1), *NDUFS2* (Hs00190020_m1) and *UXT* (Hs00188238_m1) were selected. Amplification was done as proposed by the manufacturer using the ABI 7500 system. Quantification cycle (Cq) values were determined for each sample using the comparative Cq method. Relative gene expression ratios were normalized to the reference genes 18S rRNA and hypoxanthine phosphoribosyltransferase 1 (*HPRT1*). Relative mRNA expression was represented as the fold change of the target group (marginal MCC deficiency) over the calibrator sample (control). The calibrator sample was defined as the mean expression of samples and replicates of the control group. Relative gene expression changes were calculated according to the $2^{-\Delta\Delta C_t}$ method [48,49].

Results

Urinary metabolic profiling

The metabolic screen of index patient NWU001 revealed an abnormal urinary organic acid profile with a borderline high normal level of HIVA and detectable amounts of MCG. No secondary metabolites associated with the precursor molecule, propionyl-CoA was present. The urinary acylcarnitine profile complemented the organic acid profile and revealed slightly elevated levels of 3-hydroxyisovalerylcarnitine (C5OH) and 3-methylcrotonylcarnitine (C5.1), but no severe secondary carnitine deficiency [36,50]. This patient seemed to be the first South African (Caucasian) reported with possible MCC deficiency. Molecular characterisation and further investigation was approved by the ethics committee of the North-West University (Approval number 05M 14). Fifteen family members over three generations (**Figure 1**), participated in a metabolic screen. Similar abnormal urinary organic acids and acylcarnitine urinary profiles as NWU001 were detected in three additional males in the family, the brother (NWU002) of NWU001 and grandsons of NWU002 (NWU003 and NWU004) (**Supplementary Table 1**).

In vivo L-leucine loading test

To further investigate the leucine catabolism, an *in vivo* L-leucine loading test was performed comparing the metabolite profile of the leucine degradation pathway of NWU001 and NWU002 and five healthy Caucasian adult controls. The loading test confirmed that NWU001 and NWU002 had elevated baseline levels of the urinary HIVA and MCG (**Figure 2**; **Supplementary Table 2**). After L-leucine administration, a distinct elevation of HIVA and MCG was observed in NWU001 and NWU002. The maximum level of HIVA for NWU001 was measured at T3, approximately three times higher at T3 than at T0 (T0, 37.1 and T3, 121.3 mmol/mol creatinine), whereas NWU002 had a maximum elevation of HIVA at T4, almost five times higher at T4 than at baseline (T0, 20.5 and T4, 100.4 mmol/mol creatinine). For NWU001, the levels of MCG increased more than seven times, reaching a maximum at T3 (T0, 3.1 and T3, 23.1 mmol/mol creatinine). For NWU002 the level of MCG increased almost nine times reaching a maximum at T2 (T0, 0.10 and T2, 8.6 mmol/mol creatinine). Between T4 and T5 after commencement of the L-leucine challenge, the excretion

levels of both HIVA and MCG of the two patients, NWU001 and NWU002, started to decrease. The administration of L-leucine did not affect the metabolic flux of the control participants. For the controls, the maximum level of excreted urinary HIVA was 32 mmol/mol creatinine measured at T2, which was still much lower than the baseline of NWU001. A very slight change in MCG levels in the control individuals occurred which quickly resolved, whereas NWU001 and NWU002 had detectable levels of MCG that persisted for the duration of the seven hours of monitoring.

Enzyme activities

Specific activities of MCC and PCC in fibroblasts grown in the standard FCS-based medium obtained in three or four replicate experiments are shown in **Table 1**. In fibroblasts of NWU001 and NWU002 MCC activities were slightly below or just within the reference range with mean activities reduced to 40% and 36% of the mean reference value, respectively, whereas PCC activities were within the reference range. Marginally reduced MCC activity in cells of NWU001 and NWU002 was confirmed when MCC activity was correlated with PCC activity assayed in the same cell lysate. Thus, in these two cell lines PCC/MCC activity ratios were elevated or just at the upper reference range. In contrast, MCC and PCC activities as well as the PCC/MCC activity ratios were well within the reference range in the three simultaneously assayed control cell lines.

Kinetic studies (**Table 2**) revealed that the K_m values of MCC for 3-methylcrotonyl-CoA, Na-bicarbonate and ATP as well as the requirement for the activator K^+ in cells of NWU001 and NWU002 were not importantly different from those obtained in the three simultaneously assayed control cell lines. These results exclude reduced affinity of MCC for the substrates, ATP and activator K^+ , as the cause for the marginally reduced activity of MCC in cells of the patients.

Results in **Table 3** show that the sensitivity of the fibroblasts of NWU001 and NWU002 to biotin depletion of the culture medium was not increased. Thus, after one passage in low-biotin medium MCC activity, which is more sensitive to biotin depletion than PCC activity, was reduced to 28-44% of that in high-biotin medium in cells of the patients which is similar to that seen in the three simultaneously assayed control cell lines and within the reference range. These findings exclude mild HCS deficiency as the cause for the abnormalities in the patients. However, the mean MCC activity in fibroblasts of NWU001 and NWU002 grown in the high-biotin medium was marginally reduced to 34% and 29% of the mean reference value, respectively, i.e., to similar levels as obtained in cells grown in the FCS-based medium.

The possibility of biotinidase deficiency was also excluded since the biotinidase activity of NWU001 in serum was 6.2 nmol/min/mL which is within the reference range of 3.5 -12 nmol/min/mL [43].

Mutation analyses of the open reading frames of *MCCC1* and *MCCC2*

Sequencing of *MCCC1* and *MCCC2* cDNA of NWU001 and NWU002, detected no known deleterious homozygous or compound heterozygous mutations. However, in *MCCC1* of

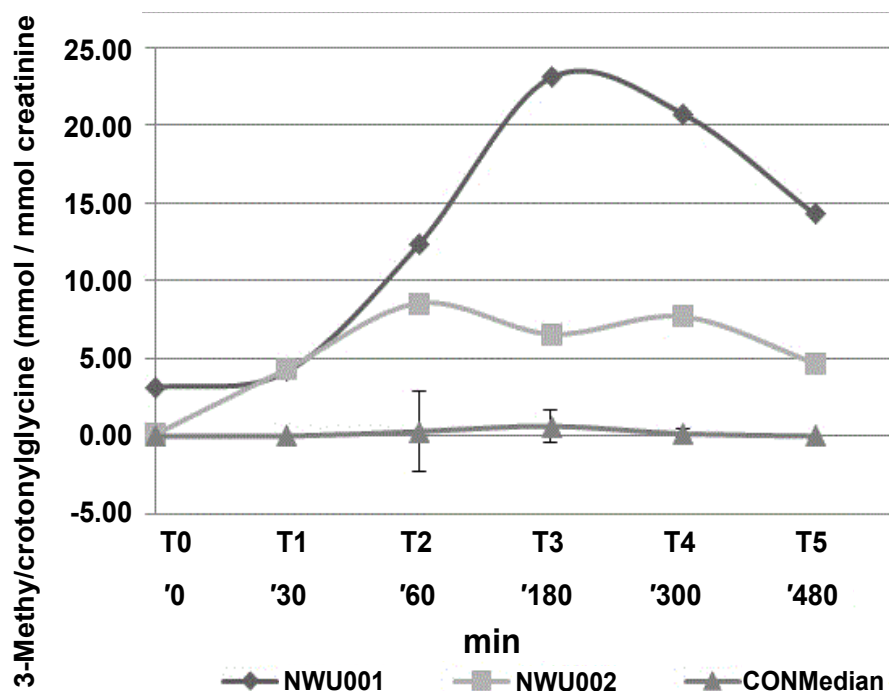


Figure 2A

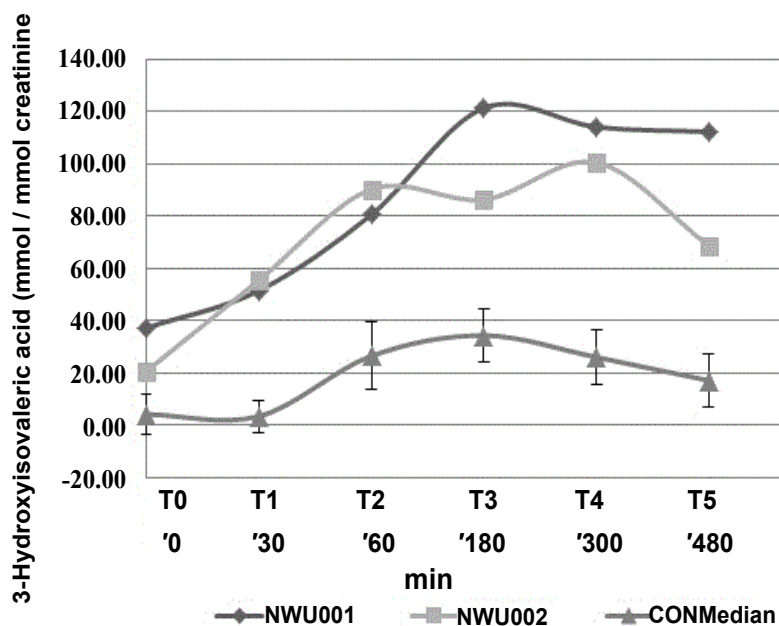


Figure 2B

Figure 2 Leucine loading test. Urinary A) 3-methylcrotonylglycine; B) 3-hydroxyisovaleric acid excretion pattern over time for NWU001, NWU002 and the median of the seven control participants.

NWU001 a heterozygous SNP (rs2270968; c.1391A>C, p.H464P) was identified and one of the *MCCC2* alleles of NWU002 contained a splice variant which resulted in the skipping of exon 7 to cause an in frame deletion of 38 amino acids (p.del209-I-A-V-V-M-G-S-C-T-A-G-G-A-Y-V-P-A-M-A-D-E-N-I-I-V-R-K-Q-G-T-I-F-L-A-G-P-P-L-246) resulting in a 525 amino acid peptide. This second peptide sequence had 100% sequence similarity to a predicted

MCCC2 isoform-2 (XM_005248624.1, Q9HCC0-2). No second mutation to suggest a homozygous or compound heterozygous genotype was detected in the ORF of either *MCCC1* or *MCCC2* of NWU002.

Whole genome expression profiling

The significantly differentially expressed transcript ID list of the marginally affected MCC deficient fibroblasts consisted of 14237 transcript IDs (**Supplementary Table 3**), of which more than 90% (13328 transcript IDs) currently have no known or only poorly defined gene associations and functional annotation. Only 1277 transcript IDs, less than 10%, have known annotation and gene association (**Supplementary Table 4**) and were included in further functional analyses. Importantly, the two MCC transcripts, *MCCC1* and *MCCC2*, were not significantly differentially expressed in the marginal MCC deficient transcriptome. To study the functional relationships, predicted network associations, biological functions, canonical pathways and toxicological associations between the 1277 well annotated transcript IDs in the dataset, IPA software (build version 242990, content version 17199142) was used.

Since the inheritance pattern of the metabolite profile in this South African family suggested a possible association with the HuChrX, the 14237 significantly differentially expressed transcript IDs were also sorted according to their chromosome association. Approximately 10% of transcript IDs have known and 90% have unknown annotations and gene associations for any particular chromosome. Of the 1277 well annotated significantly differentially expressed transcript IDs, 48 were associated with genes of HuChrX. Functional analysis of this subset was also performed separately (**Supplementary Table 5**).

Functional networks, the genetic footprint and possible implications of the marginally affected MCC deficient skin fibroblast transcriptome

The underlying molecular interactions, secondary signalling responses and functional relationships observed in the marginally affected MCC deficient skin fibroblast transcriptome were inspected following a knowledge-based functional analyses approach using IPA software. The 1277 well-annotated significantly differentially expressed transcripts indicated changes in functional networks associated with nucleic acid metabolism, cell-to-cell signalling and interaction, molecular transport, as well as cellular function and maintenance. A significant number of transcripts seemed to be involved with functional networks associated with the development of cancer (57%, 730/1277, $P = 3.83 \times 10^{-18}$) and gastrointestinal disease (21%, 268/1277, $P = 2.18 \times 10^{-4}$). Only a relatively small number of canonical pathways were significantly affected. The canonical pathway, trigger receptor expressed on myeloid cells (TREM1) was one of the canonical pathways that had a considerable number of differentially expressed transcripts in this transcriptome. TREM1 belongs to the immunoglobulin (Ig) family of cell surface receptors [51]. Activation of TREM1 triggers pathways leads to phosphorylation of signal transducers of transcription and nuclear factor kappa beta (NF- κ B), which could trigger pro-inflammatory immune responses [52]. Transcripts of the granzyme A signalling and D-myo-inositol (1,3,4)-triphosphate biosynthesis pathways were also differentially expressed. D-myo-inositol (1,3,4)-triphosphate is an important secondary messenger molecule known to

Table 1: Activities of 3-methylcrotonyl-CoA carboxylase (MCC) and propionyl-CoA carboxylase (PCC) in crude lysates of fibroblasts grown in the standard FCS-supplemented biotin sufficient medium.

Cell line*	Carboxylase activities, pmol/min/mg protein				Ratio	
	MCC		PCC		PCC/MCC	
	Individual values**	Mean	Individual values**	Mean	Individual values	Mean
NWU001	80.2, 96.8, 134, 216	132	209, 323, 483, 558	393	2.60, 3.34, 3.60, 2.58	3.03
NWU002	76.0, 95.1, 135, 165	118	286, 452, 535, 586	465	3.76, 4.75, 3.97, 3.76	4.01
CON001	329, 345, 492	389	641, 673, 1042	786	1.95, 1.95, 2.12	2.01
CON002	205, 243, 244	231	356, 493, 516	455	1.73, 2.03, 2.12	1.96
CON003	352, 404, 566	440	490, 776, 831	699	1.39, 1.92, 1.47	1.59
Reference values, range and mean						
53 different cell lines	134–671	328	208–1165	617	1.19–2.58	1.95

*Fibroblast cell lines are from the index patient (NWU001), his brother (NWU002) and 3 South African controls (CON001, CON002, CON003);

**Individual values are the mean of duplicate determinations from replicate experiments

Table 2: Km values of 3-methylcrotonyl-CoA carboxylase (MCC) for its substrates 3-methylcrotonyl-CoA (3-MC-CoA) and Na-bicarbonate as well as for ATP, and the effect of varying the concentration of the activator K^+ measured in crude fibroblast lysates.

Cell lines*	Km for 3-MC-CoA μ mol/L	Km for Na-bicarbonate mmol/L	Km for ATP μ mol/L	MCC activity, % of optimal**	
				No K^+	100 mmol/L K^+
NWU001	332	4.26	87.5	23	85.5
NWU002	560	3.49	117	25	87.5
CON001	513	4.11	128	20.6	83.7
CON002	705	3.55	116	21.1	88.9
CON003	388	4.03	94.4	16.9	93

*Fibroblast cell lines are from the index patient (NWU001), his brother (NWU002) and 3 South African controls (CON001, CON002, CON003);

**Optimal K^+ concentration (added as KCl) in the assay mixture is 50 mmol/L

Table 3: Activities of 3-methylcrotonyl-CoA carboxylase (MCC) and propionyl-CoA carboxylase (PCC) in crude lysates of fibroblasts grown in media with low and high biotin concentrations.

	Biotin in medium**	Carboxylase activities		pmol/min/mg protein	
		MCC activity***	%****	PCC activity***	%****
NWU001	Low	36.2, 55.4	28, 45	181, 398	49, 68
	High	129, 124		371, 586	
NWU002	Low	30.5, 46.5	37, 34	129, 202	50, 45
	High	82.2, 136		256, 452	
CON001	Low	76.5, 119	15, 23	405, 547	35, 52
	High	525, 520		1172, 1045	
CON002	Low	54.3	22	189	39
	High	249		485	
CON003	Low	140, 160	36, 29	377, 646	57, 56
	High	385, 557		663, 1155	
Reference values (30 different cell lines)					
Mean	Low	203	54	550	77
Range	Low	64.5-577	17-94	217-1094	33-102
Mean	High	376	-	727	-
Range	High	163-708	-	348-1514	-

*Fibroblast cell lines are from the index patient (NWU001), his brother (NWU002) and 3 South African controls (CON001, CON002, CON003);

**Fibroblasts were grown simultaneously for one passage in a medium supplemented with 10% new-born calf serum and a final biotin concentration of 0.1 nmol/L (low) and in the same medium supplemented with 10 μ mol/L biotin (high);

***Values are the mean activities of duplicate determinations from replicate experiments;

****Activity in low-biotin medium, % of activity in simultaneously assayed activity in high-biotin medium

mediate signal transduction [53] of a large number of hormones, neurotransmitters, lipid signalling as well as regulating calcium release from intracellular stores [53]. Furthermore, more than 17 upstream regulators and a significant number of their target transcripts were significantly differentially expressed. The most significantly represented upstream regulators and their associated target transcripts in the dataset were *E2F* (11 target molecules, $P = 3.40 \times 10^{-4}$), *ATF3* (4 target molecules, $P = 4.94 \times 10^{-3}$), *MAPK1* (15 target molecules, $P = 2.12 \times 10^{-2}$), *NRXN1* (5 target molecules, $P = 2.15 \times 10^{-2}$) *RNU7-1* (2 target molecules, $P = 2.47 \times 10^{-2}$) and *ELAVI* (7 target molecules, $P = 2.64 \times 10^{-2}$). The interactome of the top affected predicted upstream regulators, their association with each other and their significantly differentially expressed target transcripts were inspected (**Supplementary Figure 1**).

Functional analyses and targeted inspection of pathways and networks of interest

The marginal MCC deficiency transcriptome was further characterised by a targeted approach of inspecting selected pathways of interest overlaid with the significantly differentially expressed transcripts. The significantly differentially expressed transcripts associated with branched chain amino acid degradation, glycolysis, gluconeogenesis, tricarboxylic acid (TCA) cycle, oxidative phosphorylation system (OXPHOS), fatty acid oxidation, ketogenesis, eicosanoid signalling and xenobiotic metabolism are listed in **Table 4**. This targeted approach resulted in some key observations for the characterisation of the pathomechanism associated with marginal MCC deficiency. Only one L-leucine degradation pathway associated transcript (*HMGCL*) was significantly down regulated. The *HMGCL* gene encodes 3-hydroxy-3-methylglutaryl-CoA lyase, which catalyses

the last step of L-leucine catabolism. This enzyme (*HMGCL*) also catalyses the first step in the ketogenesis pathway, the process by which ketone bodies are produced as a result of fatty acid oxidation. Two other transcripts *HADHA* and *ALDH6A1* associated with proteins of branched chain amino acid degradation pathways were also significantly down regulated. The secondary transcriptional response also seemed to impact the glycolysis/gluconeogenesis pathway as a slight induction of *GPI* and *ME3* and down regulation of *MDH1B* was observed. One TCA associated transcript, *ACO1*, was up regulated, whereas three other transcripts, *MDH1B*, *DHTKD1*, and *SDHD* of the same pathway were down regulated. Three complexes of the OXPHOS system were affected, the *NDUFS2* of complex I and *SDHD* of complex II were down regulated, while two complex IV associated transcripts *MT-CO1/COX1* and *COX6B* were up regulated, and *COX17* was down regulated. The fatty acid oxidation-I pathway was also affected with the down regulation of *BDH2* and, as already mentioned, *HADHA* and *HMGCL*. Seven transcripts of the eicosanoid signalling pathway were differentially expressed. Three transcripts, *PLA2G2A*, *PLA2G7* and *PTGER4* were down regulated and three transcripts *PLA2G4E*, *GGT1* and *PTGIR*, were induced. Several transcripts of the xenobiotic metabolism signalling pathway specifically associated with the aryl hydrocarbon receptor (AHR) ligand activation, PXR ligand/CAR agonist activation, and MEKK signalling cascade were significantly differentially expressed (**Table 4**). Affected transcripts of the AHR activation pathway include *RELA*, *HSPA5*, *PTGES3*, *ANKRA2*, and *ALDH1A2*. Affected PXR ligand/CAR agonist activation transcripts include *CYP2C18*, *CYP2B6*, *SULT1E1*, *HS3ST1* and *MRP3/ABCC3*. Affected MEKK signalling cascade transcripts include *MAP3K* and *GCLC*.

Heat shock proteins (HSP) interact with the xenobiotic metabolism signalling via interaction through the AHR ligand activation pathway. Since HSP function was previously suggested as a possible secondary factor that could cause reduced enzyme activity of carboxylases [54], the expression of HSP was also inspected. The significantly differentially expressed transcripts of the heat shock protein interaction network were *DNAJC5*, *HSPA5*, *HSPD1*, *KIF20B*, *NSF*, *PSMC2*, *PTGES3*, *TLR3*, *TLR5*, and *TLR6* (Supplementary Figure 2).

Since oxidative stress and unregulated reactive oxygen species (ROS) were reported to contribute to MCC deficiency related neurological symptoms [55-57], the knowledge-based ROS interactome of marginal MCC deficiency was compiled and inspected. Secondary signalling responses and the associated impact on target transcripts of the interactome were identified with IPA software (Figure 3). The ROS interactome suggested activation of the NADPH oxidase complex which is associated with increased ROS production. The activated NADPH oxidase complex and elevated ROS levels also impact on the pro-inflammatory

cytokine regulatory system which involves the regulation of PLA_2 , $NF\kappa B$ and Hsp70 interaction networks. The persistent activation of NADPH oxidase and increased ROS production seemed to cause chronic activation of TNF- α and other immune and inflammatory related response pathways.

Functional analysis of the HuChrX transcript subset

To better understand the specific contribution of HuChrX associated transcripts to the marginal MCC deficiency transcriptome, the subset of 48 significantly differentially expressed HuChrX associated transcripts were inspected separately. Functional network analysis showed that these 48 HuChrX associated transcripts clustered mainly in two independent functional networks (Figures 4a and 4b; Supplementary Table 6). The first network included molecules functionally important for molecular and cellular functions that involve cellular assembly, organisation, optimal function, and maintenance ($P = 5.03 \times 10^{-4} - 4.74 \times 10^{-2}$). The second functional network predominantly

Table 4: Important canonical pathways and associated significant differentially expressed transcripts in the marginal 3-methylcrotonyl-CoA carboxylase deficient skin fibroblast transcriptome.

Canonical Pathway	Gene name/description	Gene Symbo	P Value	Fold change	*	Entrez ID
Branched chain amino acid degradation						
Isoleucine degradation	Hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase (trifunctional protein), alpha subunit	HADHA	4.17E-02	-1.39		3030
Leucine degradation	3-hydroxymethyl-3-methylglutaryl-CoA lyase	HMGCL	1.99E-02	-1.13		3155
Valine degradation	Hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase (trifunctional protein), alpha subunit	HADHA	4.17E-02	-1.39		3030
	Aldehyde dehydrogenase 6 family, member A1	ALDH6A1	4.64E-02	-1.29		4329
Cholesterol biosynthesis						
	Sterol-C5-desaturase	SC5DL	4.19E-02	-1.26		6309
Eicosanoid biosynthesis						
	Phospholipase A2, group IVE	PLA2G4E	2.76E-03	1.10		123745
	Phospholipase A2, group IIA (platelets, synovial fluid)	PLA2G2A	6.89E-03	-1.31		5320
	Phospholipase A2, group VII (platelet-activating factor acetylhydrolase, plasma)	PLA2G7	1.18E-02	-1.09		7941
	Gamma-glutamyltransferase 1	GGT1	1.72E-02	1.32		2678
	Prostaglandin I2 (prostacyclin) receptor (IP)	PTGIR	2.84E-02	1.23		5739
	Prostaglandin E receptor 4 (subtype EP4)	PTGER4	4.44E-02	-1.35		5734
Fatty acid alpha oxidation						
	Aldehyde dehydrogenase 1 family, member A2	ALDH1A2	1.22E-02	1.21		8854
Fatty acid beta oxidation						
	Hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA Thiolase/enoyl-CoA hydratase (trifunctional protein), alpha subunit	HADHA	4.17E-02	-1.39		3030
Gluconeogenesis						
	Malic enzyme 3, NADP(+)-dependent, mitochondrial	ME3	8.19E-03	1.07		10873
	Glucose-6-phosphate isomerase	GPI	3.02E-02	1.18		2821
	Malate dehydrogenase 1B, NAD (soluble)	MDH1B	4.77E-02	-1.11		130752
Glycolises						
	Glucose-6-phosphate isomerase	GPI	3.02E-02	1.18		2821

Heat Shock Protein interaction						
	DnaJ (Hsp40) homolog, subfamily C, member 5	DNAJC5	7.00E-03	1.20		80331
	Heat shock 70kDa protein 5 (glucose-regulated protein, 78kDa)	HSPA5	4.91E-02	1.69		3309
	Heat shock 60kDa protein 1 (chaperonin)	HSPD1	6.15E-03	-1.64		3329
	Kinesin family member 20B	KIF20B	2.11E-02	-1.20		9585
	N-ethylmaleimide-sensitive factor	NSF	1.97E-02	-1.11		4905
	Proteasome (prosome, macropain) 26S subunit, ATPase, 2	PSMC2	4.14E-03	-1.44		5701
	Prostaglandin E synthase 3 (cytosolic)	PTGES3	2.81E-02	-1.75		10728
	Toll-like receptor 3	TLR3	4.68E-02	-1.36		7098
	Toll-like receptor 5	TLR5	2.97E-02	1.99		7100
	Toll-like receptor 6	TLR6	3.95E-02	-1.62		10333
Ketogenesis						
	3-hydroxymethyl-3-methylglutaryl-CoA lyase	HMGCL	1.99E-02	-1.13		3155
	3-hydroxybutyrate dehydrogenase, type 2	BDH2	3.47E-02	-1.59		56898
	Hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase (trifunctional protein), alpha subunit	HADHA	4.17E-02	-1.39		3030
Oxidative phosphorylation						
	Cytochrome c oxidase subunit I	COX1	2.04E-02	1.34		4512
	COX17 cytochrome c oxidase copper chaperone	COX17	3.07E-02	-2.17		10063
	Succinate dehydrogenase complex, subunit D, integral membrane protein	SDHD	3.29E-02	-1.17		6392
	Cytochrome c oxidase subunit VIb polypeptide 1 (ubiquitous)	COX6B1	3.97E-02	1.31		1340
	NADH dehydrogenase (ubiquinone) Fe-S protein 2, 49kDa (NADH-coenzyme Q reductase)	NDUFS2	4.83E-02	-1.22		4720
Tricyclic acid cycle						
	Dehydrogenase E1 and transketolase domain containing 1	DHTKD1	3.12E-02	-1.16		55526
	Succinate dehydrogenase complex, subunit D, integral membrane protein	SDHD	3.29E-02	-1.17		6392
	Aconitase 1, soluble	ACO1	4.48E-02	3.59		48
	Malate dehydrogenase 1B, NAD (soluble)	MDH1B	4.77E-02	-1.11		130752
Xenobiotic metabolism signaling						
AHR signaling	Aldehyde dehydrogenase 1 family, member A2	ALDH1A2	1.22E-02	1.21		8854
	Aldehyde dehydrogenase 6 family, member A1	ALDH6A1	4.64E-02	-1.29		4329
	Ankyrin repeat, family A (RFXANK-like), 2	ANKRA2	1.95E-02	-1.15		57763
	Heat shock 70kDa protein 5 (glucose-regulated protein, 78kDa)	HSPA5	4.91E-02	1.69		3309
	Prostaglandin E synthase 3 (cytosolic)	PTGES3	2.81E-02	-1.75		10728
	v-rel avian reticuloendotheliosis viral oncogene homolog A	RELA	4.83E-03	-1.32		5970
		TEBP				
CAR-PXR signaling	ATP-binding cassette, sub-family C (CFTR/MRP), member 3	ABCC3/MRP3	4.08E-02	1.17		8714
	Cytochrome P450, family 2, subfamily B, polypeptide 6	CYP2B6	4.23E-02	1.21		1555
	Cytochrome P450, family 2, subfamily C, polypeptide 18	CYP2C18	1.26E-02	-1.19		1562
	Sulfotransferase family 1E, estrogen-preferring, member 1	SULT1E1	4.24E-02	-1.84		6783
	Heparan sulfate (glucosamine) 3-O-sulfotransferase 1	HS3ST1	4.01E-02	-1.36		9957
MEKK signaling	Glutamate-cysteine ligase catalytic subunit	GCLC	2.31E-02	-1.31		2729
	Mitogen-activated protein kinase kinase kinase 6	MAP3K6	9.60E-03	-1.21		9064

*Heat map: Red indicates up regulated transcripts; Green indicates down regulated transcripts

included molecules involved with neurological development ($P = 6.52 \times 10^{-4} - 4.74 \times 10^{-2}$) and lipid metabolism ($P = 1.51 \times 10^{-3} - 9.05 \times 10^{-3}$). The most affected canonical pathways indicated by the HuChrX transcripts were GABA receptor signalling, anandamide degradation and glycogen biosynthesis II from UDP-D glucose pathways. One of the most interesting findings of the differentially expressed HuChrX associated transcript analyses was the 1.28 fold induction of the *FAAH2* transcript. *FAAH2* is one of two transcripts that code for fatty acid amide hydrolases, the other transcript is *FAAH*. These enzymes convert fatty acid amides to ethylamine and the corresponding fatty acid. While *FAAH* seems to prefer poly-unsaturated fatty acid amides as substrates, it seems as if *FAAH2* is more specific for mono-unsaturated fatty acid amides [58]. However, *FAAH2* is not yet well characterised. It is important to note that induction of *FAAH/FAAH2* has been reported to be associated with pro-inflammatory signalling [59].

Independent qPCR validations

The HuExST1.0 array gene expression data was validated with independent qPCR analysis of eight selected significantly differentially expressed transcripts, *HADHA*, *HMGCL*, *ACO2*, *AWAT2*, *NDUFS2* and *UXT* using TaqMan hydrolysis probe chemistry gene expression assays. The fold change of the six selected differentially expressed transcripts from the HuExST1.0 array experiment was compared to the fold change obtained with the independent qPCR analyses. The data from triplicate experiments were analysed and the expression ratios of genes between the two groups (marginal MCC deficiency and Controls) were calculated using the $2^{-\Delta\Delta Ct}$ method [48,49]. The expression profiles of these two independent datasets confirmed one another (Supplementary Figure 3).

Discussion

Here we present results of an investigation of the first evidence of MCC deficiency detected in four males in South Africa. The metabolite profile, indication of heritability, enzyme studies and *in vivo* L-leucine loading suggested that they have marginal MCC deficiency. Overall, the biochemical, enzymatic and genetic profile of NWU001 and NWU002 seemed similar to that of a group of previously described patients with partially reduced MCC activity [17]. The increased PCC/MCC ratio for NWU001 and NWU002 was similar to that reported for patients with elevated metabolites indicative of MCC deficiency and only one mutation in either *MCCC1* or *MCCC2* [17,19]. Sequencing of the coding region revealed that NWU001 and NWU002 did not share a common MCC genotype. NWU001 and NWU002 only presented with one heterozygous SNP and did not have a second mutation in either *MCCC1* or *MCCC2*. These results are similar to a previous report wherein it was suggested that only one mutant allele in the presence of a wild type allele could also possibly explain the biochemical phenotype indicative of MCC deficiency [17]. Follow up studies are needed to further investigate the functional characteristics of the shorter *MCCC2* isoform-2 detected in NWU002, which lacks exon 7, to better understand the impact of this heterozygous genetic variation.

In addition to the usual biochemical work-flow, we generated a transcriptome from cultured skin fibroblasts of the two

marginal MCC deficient South African patients, NWU001 and NWU002, and two control adult individuals. We inspected the differentially expressed transcripts and secondary signalling responses for clues to the pathomechanism that might affect the MCC deficiency phenome. Two important observations from this transcriptome support the notion that secondary factors other than the MCC loci impact on the presentation of MCC deficiency. Firstly, both MCC transcripts, *MCCC1* and *MCCC2*, were not significantly differentially expressed. This is in contrast to the transcriptome of symptomatic, clinical severely affected cases of MCC deficiency where *MCCC1* and *MCCC2* were significantly differentially expressed. Secondly, the finding that the vast majority (>90%) of the significantly differentially expressed transcripts are still poorly annotated also seem to suggest a role of as yet unknown transcripts in this disorder.

Although the number of arrays analysed in this study was very limited and could raise concern for over interpretation and bias, the underlying molecular interaction networks and associated differentially expressed transcripts confirm and extend results from various previous studies on MCC deficiency. Similar to the transcriptome of symptomatic, clinically severe MCC deficiency and neurological studies on the cerebral cortex of young rats [55-57,60-65], this marginal MCC deficiency skin fibroblast transcriptome also had a genetic footprint suggestive of partial mitochondrial dysfunction, oxidative stress and disruption of energy homeostasis. Transcripts of mitochondrial respiratory chain complexes I, II and IV were significantly differentially expressed (Table 4) affecting the OXPHOS system (*NDUFS2*, *SDHD*, *MT-CO1/COX1*, *COX6* and *COX17*). This result correlates with the finding that the metabolites, MCG and MCA, inhibited mitochondrial respiratory chain complexes II and III *in vitro* in rat brain homogenates [55] and could induced lipid and protein oxidative damage [55,57,64].

Aconitase 1 (*ACO1*), a hypoxia and oxidative stress sensitive transcript associated with TCA, was induced, an indication of elevated ROS and increased oxidative stress [66,67]. The NADPH oxidase complex was also induced, another indication of increased hydrogen peroxide, superoxide, hydroxyl radical, and oxygen radicals production and elevated levels of peroxynitrite [68]. Figure 3 shows the relationship between the induced NADPH oxidase activity, increased ROS production and activation of the pro-inflammatory responses via the eicosanoid biosynthesis pathway [68,69]. The induction of the NADPH oxidase complex usually induces NF κ B signalling [68], but this seems not to be the case in this transcriptome since *RELA*, a transcript coding for a protein of the NF κ B complex was down regulated and thus seem to regulate the NF κ B signalling pathway. This suppression of the NF κ B complex also impacts on the aryl hydrocarbon receptor (AHR) signalling cascade of the xenobiotic metabolism signalling and regulates the expression of genes involved in the innate and adaptive immune response.

Several transcripts that affect the AHR activation pathway and HSP networks were significantly differentially expressed. The significantly altered HSP network included the Hsp70 network (*HSPA5*) and ATPase (*HSPD1*), but not transcripts of Hsp60, which were reported to possibly contribute to phenotypic differentiation in MCC deficiency [54,70]. The HSP interaction network (Supplementary Figure 2) also showed significant differentially expressed Toll-like receptors which could further

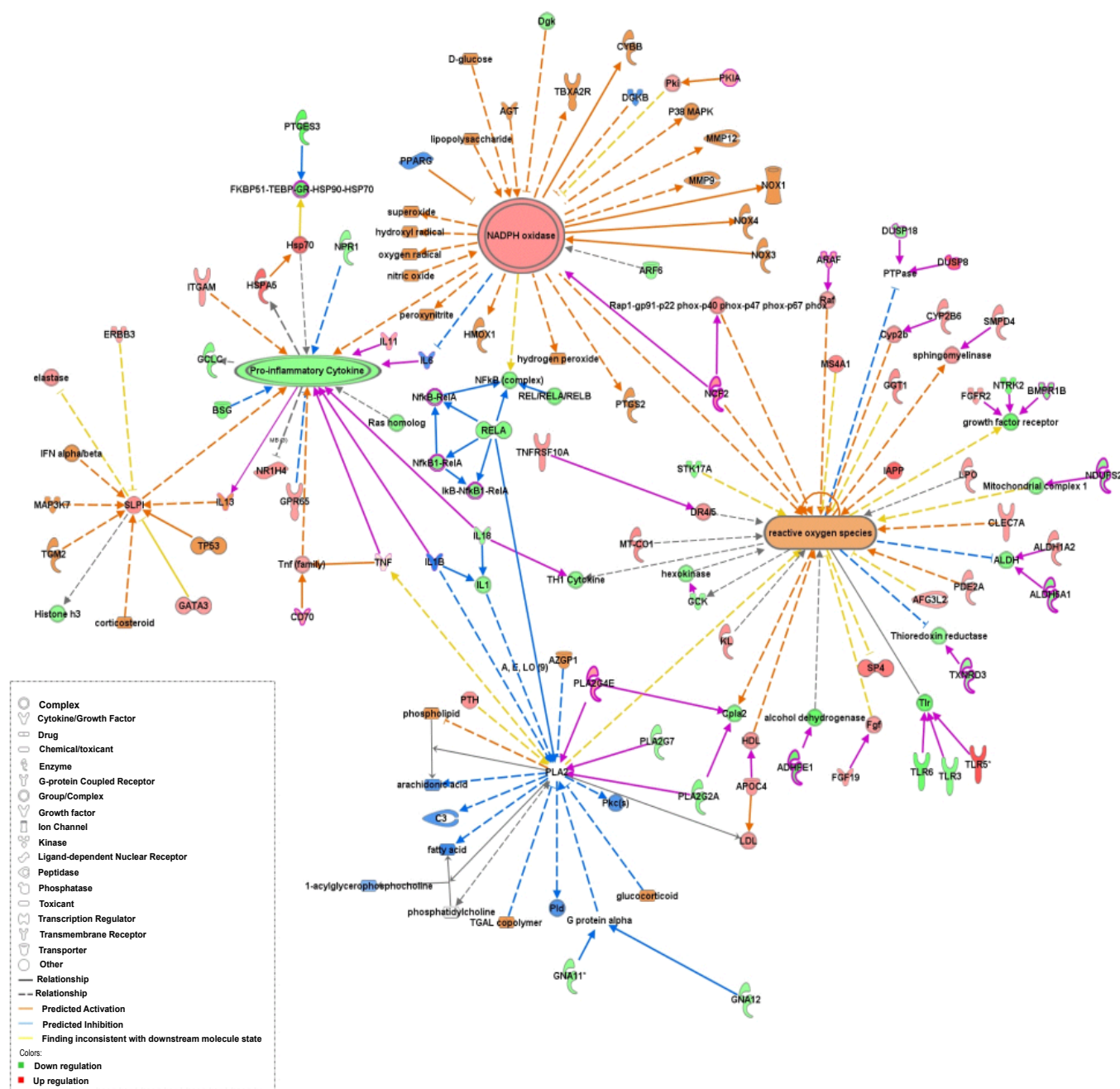
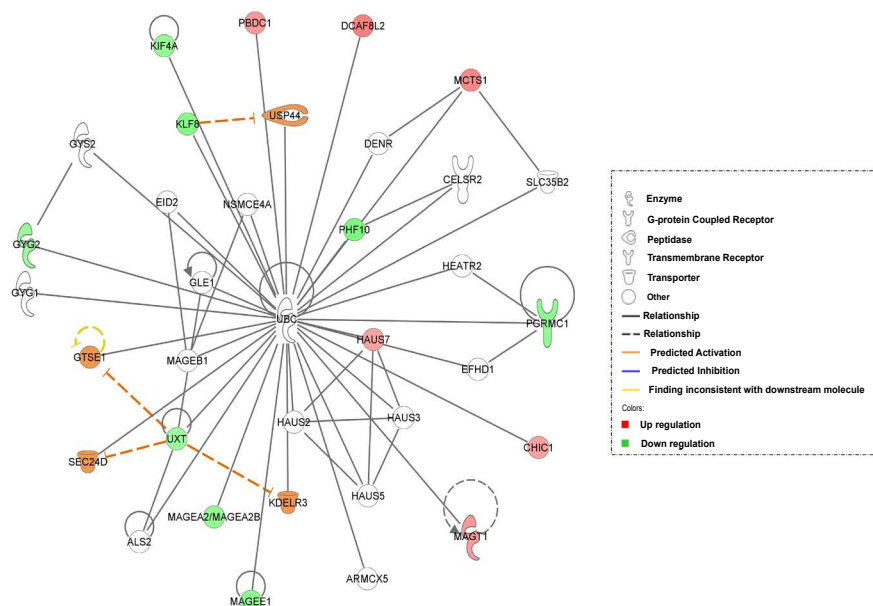


Figure 3 Graphic representation of the marginal MCC deficiency ROS interactome. Graphical representation of the implicated ROS interactome overlaid with the significantly differentially expressed transcripts. Red indicates up regulated, green down regulated and white molecules not significant differentially expressed but essential to network interaction. Orange suggests predicted activation and blue predicted inhibition interactions.

influence the inflammatory signalling as well as activation of the NF κ B and TNF α inflammatory signalling cascades that can add to the oxidative stress burden [71,72]. This marginal MCC deficiency ROS interactome suggests chronic activation of inflammatory and immune signalling responses. Chronic activated of pro-inflammatory signalling has been associated with compromised cellular membrane integrity and the development of cancer [69,73], Parkinson's disease [74-76], Alzheimer's disease [74,75,77-80], rheumatoid arthritis [59,81] and cardiovascular disease [61,82].

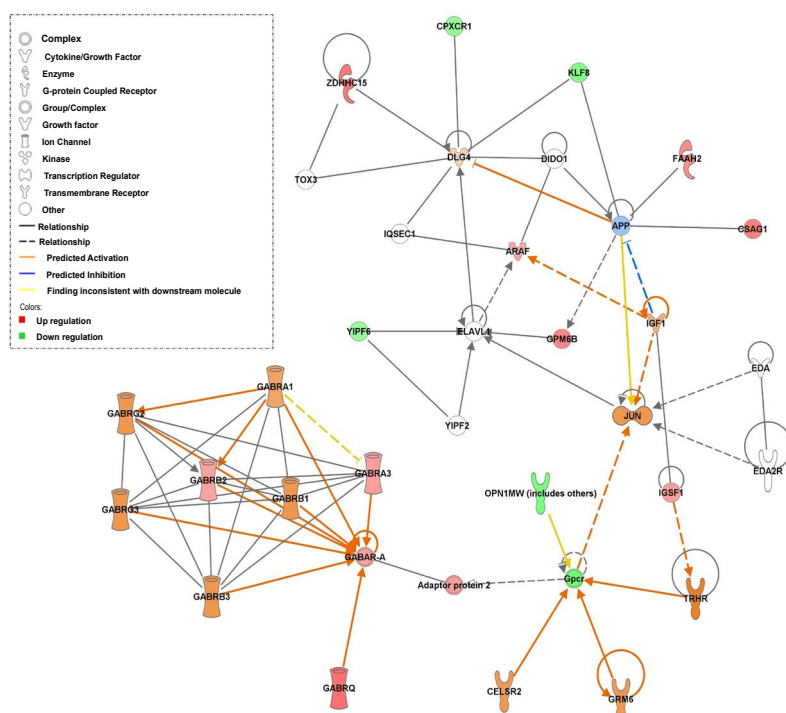
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Both the complete set of 1277 and the subset of 48 HuChrX associated differentially expressed transcript IDs indicated increased inflammatory signalling responses and impaired cellular maintenance and repair activity that could imply compromised membrane integrity (**Figure 4a; Supplementary Table 6**). It is important to note that persistent oxidative stress in addition to an already compromised membrane repair capacity could severely affect membrane integrity. Membrane integrity homeostasis is highly regulated and a complex machinery that impact on



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Figure 4A



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Figure 4B

Figure 4 Predicted functional networks for the human chromosome-X. Graphical 60 representations of predicted functional relationships overlaid with the significant differentially expressed 61 HuChrX associated transcripts. Red indicates up regulated, green showed down regulated and white 62 molecules not significant differentially expressed but essential to network interaction. Orange suggests 63 predicted activation and blue predicted inhibition interactions. A) Network 1: Shows a network 64 interaction of transcripts involved with cellular function and maintenance; B) Network 2: Suggesting 65 activation of the GABA receptor signaling pathway and involvement with neurological disease.

lipid and bioactive lipid signalling through the activation of a phospholipase interaction network [59]. A striking and a perhaps important observation that could aid elucidating the underlying X-linked contribution to marginal MCC deficiency, was the significant up regulation of the *FAAH2* (fatty acid amide hydrolase variant 2), a transcript of the anandamide degradation canonical pathway. The control of *FAAH/FAAH2* expression was reported to have promising therapeutic value to control inflammatory related clinical symptoms [59]. Induction of the *FAAH2* transcript implies a possible activation of the hydrolase activity converting anandamide to arachidonic acid (AA) and ethanolamide and thus contributes to the activation of the biosynthesis of pro-inflammatory bioactive lipids [59]. The overexpression of fatty acid amide hydrolases can compromise innate immunity in *Arabidopsis* [83]. Furthermore, inhibition of *FAAH/FAAH2* seems to normalize cardiovascular function in hypertensive rats as well as normalise endotoxin-induced enhanced gastrointestinal motility in mice [84]. Strong associations between aberrant regulation of *FAAH/FAAH2* and alcohol and substance dependency, as well as obesity [85] have been made. The overexpression of *FAAH/FAAH2* also associates with sleep deprivation [58] as well as cholesterol level maintenance [86]. Incidentally, the index patient NWU001 experienced a number of symptoms which seem to correlate with the transcriptome footprint and abovementioned literature. Another reason for the specific interest in *FAAH2* with regard to the unravelling of the underlying molecular pathomechanism of MCC deficiency is that NWU001 and NWU002 both had notably high blood triglycerides levels but only slightly reduced HDL, normal LDL and borderline elevated cholesterol levels. This might be a coincidence, but if not, could be an indicator to predict and monitor the development of disease and should be further investigated.

A number of phospholipase family 2 (PLA_2) transcripts were also differentially expressed. These enzymes have multiple functions in membrane phospholipid maintenance, homeostasis and the production of lipid mediators [87]. The PLA_2 family releases fatty acids from the *sn*-2 position of the membrane phospholipids [88]. Since the *sn*-2 position of phospholipids consists mainly of AA, docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), fatty acids are released to the cell [80] by the activation of specific PLA_2 enzymes. Increased cellular AA enables the bioactive lipid signalling metabolism to synthesise pro-inflammatory eicosanoids [87] or be recycled into phospholipid by an acyltransferase [89]. The PLA_2 family consists of six subtypes of which cytosolic PLA_2 (c PLA_2) is AA specific, whereas calcium-independent PLA_2 (i PLA_2) seems to be DHA specific [80,82]. The marginal MCC deficiency transcriptome, however, showed differential expression of the c PLA_2 transcripts *PLA2G2A*, *PLA2G4E*, and *PLA2G7* which suggests inhibition of the c PLA_2 machinery, probably in an attempt to regulate the pro-inflammatory signalling. A prolonged inflammatory state causes cell damage and is associated with the pathogenesis of many diseases [90,91]. Also, c PLA_2 seems to play a major role in lipid class switching [92] and is therefore considered as an important decision maker for regulating inflammation. The acute inflammatory process stays unresolved and develops into chronic inflammation when the pro-resolving mediators are not formed or are not functioning correctly. Significant differential

expression of other eicosanoid biosynthesis pathway related transcripts *GGT*, *PTGIR*, *PTGER4* and *PTGES3* was also observed. The character of these transcripts predicted activation of *PTGES2* (cyclooxygenase-2, COX-2), which suggests activation of the lipid mediator biosynthesis pathway. The enzymes encoded by *PTGES* genes are both pro- and anti-inflammatory lipid mediators. However, the induction of *GGT* suggests elevated levels of leukotriene D4 and the suppression of *PTGES3* suggests elevated levels of prostacyclin which both have strong pro-inflammatory properties [93]. Imbalances in eicosanoid metabolism signalling have been detected in individuals with a weak tolerance to environmental factors and susceptibility to environmental stressors that often result in asthma [94-97], allergy, eczema [98-100], arthritis [81] as well as the development of cancer [73] and might also play a role in the development of non-specific symptoms of marginal MCC deficiency. Therefore, life-long monitoring of ROS levels of individuals with MCC deficiency along with the administration of anti-oxidants to prevent and manage deleterious secondary signalling responses may be beneficial as previously proposed (**Figure 3**) [55,56,60,64,65,101].

The apparent X-linked inheritance of urinary HIVA and MCG in this family was intriguing because MCC deficiency is an autosomal recessive inherited disease and there are no reports on an X-linked association. Another indication of a possible HuChrX-link in MCC deficiency was the report wherein six of eight patients who presented with metabolites of MCC deficiency, but for whom no MCC mutations could be detected were males [17]. The incidence of 3-methylglutaconic aciduria Type-I, another autosomal recessive disorder of the leucine catabolism, also seems to be higher in males than in females [16,102]. The apparent HuChrX link in both MCC and MCGA-Type-I deficiencies could be coincidental, but should be further investigated. Independent analyses of the subset of 48 HuChrX transcripts showed a significant impact on the GABA interactome. Although GABA receptors and signalling are tissue-specific, the activation and suppression of some of the GABA receptors specific for muscle function, organ and brain health in this skin fibroblast transcriptome and could possibly underlie some of the non-specific, mild psychological and muscle related symptoms experienced by the index patient NWU001. Aberration of GABA signalling pathways is well characterised for neuron cells and associated with the development of psychological disease [103].

Upstream regulators with the most significant overlap between their target molecules and the significantly differentially expressed transcripts were closely inspected. Interestingly, several downstream target genes of these regulatory factors are located on HuChrX. The upstream regulator E2F had the most significant representation of target transcripts in this transcriptome, two of which are located on HuChrX. The progesterone receptor membrane component 1 (*PCRM1*) is a protein binding transmembrane receptor that regulates cholesterol amongst other molecules. Another HuChrX encoded target molecule of the upstream E2F transcription regulator is the ubiquitously-expressed, prefolding-like chaperone (*UXT*). This protein functions as a cofactor that modulates androgen receptor dependent transcription (**Supplementary Figure 1**).

Conclusion

In conclusion, this first investigation of marginal MCC deficiency in South Africa was initiated when NWU001 and three other male family members presented with urinary HIVA and MCG. The marginal MCC deficiency skin fibroblast transcriptome had a footprint of oxidative stress, partial mitochondrial dysfunction, disruption of energy homeostasis, inflammation, and impaired cellular maintenance and repair mechanisms, similar to what was observed in the transcriptome of clinically severe MCC deficient skin fibroblasts [104]. The functional analyses indicated that although MCC deficiency is considered a non-disease with little clinical impact, the secondary signalling responses predict a chronic activation of pro-inflammatory signalling and ROS production which could result in a detrimental long-term effect and should therefore be controlled. The observations that the two MCC transcripts, *MCCC1* and *MCCC2*, were not significantly differentially expressed and that more than 90% of the significantly differently expressed transcripts are still poorly annotated, makes it impossible to come to firm conclusions and further support the notion that secondary factors other than the MCC loci impact on the MCC deficiency phenotype.

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Conflict of Interest

Lizelle Zandberg, Lardus Erasmus, Carina Mels, Terttu Suormala, Japie Mienie, and Alberdina van Dijk declare that they have no conflict of interest.

Informed Consent

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000 (5). Informed consent was obtained from all patients for being included in the study.

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