Human Pigmentation, Cutaneous Vitamin D Synthesis and Evolution: Variants of Genes (SNPs) Involved in Skin Pigmentation Are Associated with 25(OH)D Serum Concentration

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Abstract. *Vitamin D deficiency is common and associated with higher risk for and unfavourable outcome of many diseases. Limited data exist on genetic determinants of serum 25(OH)D concentration. In a cohort of the LURIC study (n=2974, median 25(OH)D concentration 15.5 ng/ml), we tested the hypothesis that variants (SNPs, n=244) of several genes (n=15) involved in different aspects of skin pigmentation, including melanosomal biogenesis (ATP7A, DTNBP1, BLOC1S5, PLDN, PMEL), melanosomal transport within melanocytes (RAB27A, MYO5A, MLPH); or various melanocyte signaling pathways (MC1R, MITF, PAX3, SOX10, DKK1, RACK1, CNR1) are predictive of serum 25(OH)D levels. Eleven SNPs located in 6 genes were associated ($p<0.05$) with low or high serum 25(OH)D levels, 3 out of these 11 SNPs reached the aimed significance level after correction for multiple comparisons (FDR). In the linear regression model adjusted for sex, body mass index (BMI), year of birth and month of blood sample rs7565264 (MLPH), rs10932949 (PAX3), and rs9328451 (BLOC1S5) showed a significant association with 25(OH)D. The combined impact on variation of 25(OH)D serum levels (coefficient of determination (R^2)) for the 11 SNPs was 1.6% and for the 3 SNPs after FDR 0.3%. In Cox Regression we identified rs2292881 (MLPH) as having a significant association (advantage) with overall survival. Kaplan-Meier analysis did not show any significant impact of individual SNPs on overall survival. In conclusion, these results shed new light on the role of sunlight, skin pigmentation and vitamin D for human evolution.*

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Key Words: Human pigmentation, vitamin D, SNPs, melanocytes, sunlight.

Vitamin D deficiency represents a major health problem in the Caucasian population (1). The need to obtain adequate sources of vitamin D has been identified as an important driver of human evolution (2). Vitamin D regulates the absorption of calcium, and humans need vitamin D to develop and maintain a healthy mineralized skeleton (3). More recently, it has been shown that vitamin D status is of importance for prevention and outcome of a broad variety of diseases that are unrelated to bone and calcium metabolism (3). Many tissues (*e.g.* skin, skeletal muscle, brain, prostate, breast, colon) and cell types (*e.g.* immune cells) express the vitamin D receptor (VDR) and have been identified as targets of the biologically-active vitamin D metabolite 1,25-dihydroxyvitamin D_3 ($1,25(OH_2)D_3$) (3). It has been shown that low serum 25(OH)D concentrations are associated with an unfavorable outcome of and an increased risk for many diseases, including various types of cancers, infectious, autoimmune and cardiovascular diseases (3). In the human body, vitamin D is either absorbed dietetically or synthesized from 7-dehydrocholesterol (7-DHC) in the presence of ultraviolet B (UVB) radiation in the skin (3). Vitamin D is metabolized in the liver by CYP2R1 (cytochrome *P450* 2R1) or CYP27A1 (cytochrome *P450 27A1*) to 25-hydroxyvitamin D (25(OH)D), which is the major circulating metabolite and

Table I. *SNPs with relevance for skin pigmentation and vitamin D status: Short description of genes investigated in this study*

Gene	Gene product	Gene name	Localization	Function
ATP7A	Copper-transporting ATPase 1	ATPase, Cu^{++} transporting, alpha polypeptide	Xq21.1	Copper transporter in the trans-golgi network
BLOC1S5	muted	biogenesis of lysosomal organelles complex-1, subunit 5, muted	6p25.1-p24.3	BLOC-1 subunit
CNR1	CNR1	cannabinoid receptor 1	6q14-q15	Receptor for cannabinoids, induces melanin synthesis
DKK1	DKK1	dickkopf 1	10q11.2	Inhibits proliferation of melanocytes *via* inhibition of WNT signaling
DTNBP1	DTNBP1	dystrobrevin binding protein 1	6p22.3	BLOC-1 subunit
MC1R	MC1R	melanocortin-1 receptor	16q24.3	Regulates production of eumelanin *versus* pheomelanin
MITF	MITF	microphtalmia-associated transcription factor	3p14.1-p12.3	Contributes to signal transduction in melanocytes
MLPH	MLPH	melanophilin	2q37.2	Transport of melanosomes
MYO5A	MYO5A	myosin VA (heavy chain 12, myoxin)	15q21	Transport of melanosomes
PAX3	PAX3	paired box 3	2q36.1	Transcription factor
PLDN	pallidin	biogenesis of lysosomal organelles complex-1, subunit 6, pallidin	15q21.1	BLOC-1 subunit
PMEL	PMEL	premelanosome protein	12q13-q14	Main structure component of melanosomes
RAB27A	RAB27A	RAB27A, member RAS oncogene family	15q15-q21.1	Transport of melanosomes
RACK1	GNB2L1	guanine nucleotide binding protein (G protein), beta polypeptide 2-like 1	5q35.3	Regulator of the signal transduction in the melanocyte
SOX10	SOX10	SRY (sex determining region Y)-box 10	22q13.1	Transcription factor

the best marker for the vitamin D status of an individual (3). The biologically active form of vitamin D is 1,25-dihydroxyvitamin D ($1,25(OH)_2D$), which is generated from 25(OH)D in the kidney or in other tissues by CYP27B1. In target cells $1,25(OH)_2D$ is catabolized by $1,25(OH)_2D$-24-hydoxylase (CYP24A1) to water-soluble, biologically inactive calcitroic acid, which is excreted through the bile (3).

Vitamin D deficiency is very common and represents a major health problem in the Caucasian population (1). Consequently, the identification of people at-risk to develop vitamin D deficiency and the search for factors that predispose for a person' s vitamin D status is of high scientific interest. Limited data have been provided on genetic determinants of serum 25(OH)D concentration. A genome-wide association study (GWAS) identified three loci, that are associated with the vitamin D status (4). They are located in genes encoding for proteins related to vitamin D transport (group-specific component, GC), cholesterol synthesis (DHCR7) and hydroxylation of vitamin D metabolites (CYP2R1, CYP24A1) (4). However, genes related to skin pigmentation have not been found in this analysis, although skin type has been previously identified in numerous clinical and epidemiological studies to predispose for a person's individual vitamin D status (5). It is well-known that limitations of GWAS, including conservative data analysis and pooling data from heterogeneous cohorts, may result in a type II error, missing existing associations. Therefore, we decided to investigate whether variants of genes involved in skin pigmentation are associated with vitamin D status in a well-defined cohort of Caucasians. The pigmentation of the skin is a complex process involving various hormones, corresponding receptors and other factors (6). In a first pilot study, we identified the association of several genes related to skin pigmentation, including exocyst complex component 2 (*EXOC2*), tyrosinase (*TYR*), tyrosinase-related protein 1 (*TYRP1*) and dopachrome tautomerase (*DCT*) with vitamin D status (2). To gain further insight into the role of genes involved in skin pigmentation we analyzed whether single nucleotide polymorphisms (SNPs) in 15 other genes that are of high importance for skin pigmentation (Table I), are associated with serum 25(OH)D concentration. Our cohort includes 2,974 caucasians derived from the Ludwigshafen Risk and Cardiovascular Health (LURIC) study.

Patients and Methods

SNPs (n=244) of genes (n=15) involved in skin pigmentation and 25(OH)D serum concentrations, were retrospectively analyzed in a cohort of Caucasians (n=2,974) derived from the LURIC study (7).

Ludwigshafen Risk and Cardiovascular Health (LURIC) study. The LURIC study has been described in detail previously (7). In short, it consists of 3,316 Caucasian patients hospitalized for coronary

angiography between 1997 and 2000 at the Ludwigshafen General Hospital in Southwestern Germany. This ongoing prospective cohort study investigates environmental, biochemical and genetic risk factors for coronary artery disease (CAD) (7). Angiography was performed in patients with chest pain or a positive non-invasive stress test suggestive of myocardial ischemia. CAD has been defined as the presence of a visible luminal narrowing (>20% stenosis) in ≥1 of 15 coronary segments according to a classification of the American Heart Association. Informed written consent was obtained from all participants. The study was approved by the ethics committee at the "Ärztekammer Rheinland-Pfalz" and was conducted in accordance with the Declaration of Helsinki (7, 8). The design of our present study is outlined in Figure 1.

Laboratory procedures. Laboratory procedures have been described in detail previously (8). In short, venous blood was obtained from the patients in the early morning by venipuncture after an overnight fasting period. Genomic DNA was prepared from EDTA-anticoagulated blood using a common salting-out procedure (8).

Within 30 min the remaining blood was centrifuged at 3,000 × *g* for 10 min, immediately aliquoted and frozen at –80˚C until further analysis. Serum levels of 25(OH)D were measured with a radioimmunoassay (RIA) (DiaSorin SA, Antony, France, intra-assay and inter-assay coefficients of variation of 8.6% and 9.2%). $1,25(OH)_2D$ serum concentrations were also measured by radioimmunoassay (Nichols Institute Diagnostika GmbH, Bad Nauheim, Germany) on a multicrystal counter (Berthold LB2014, DiaSorin, SA, USA) (9). Intact PTH was determined in serum by ElectroChemiLuminescence Immunoassay (ECLIA) on an Elecsys 2010 (Roche Diagnostics, Mannheim, Germany), with a normal range of 15-65 pg/mL and an inter-assay coefficient of variation of 5.7-6.3% (9).

Genotyping and quality control (QC). Genotyping and quality control have been described in detail previously (8). In short, genotyping was performed using the Affymetrix Human SNP Array 6.0 at the LURIC facility (Heidelberg, Germany). Genotype imputation was performed using MACH 1.0 and HapMap II CEU (release 22, NCBI build 36, dbSNP 126) samples as a reference. After imputation, 2,543,887 SNPs were available. SNPs with squared correlation of ≥0.3 between imputed and true genotypes were considered well imputed (8).

From the aforementioned dataset, SNPs within genes ATPase, Cu^{++} transporting, alpha polypeptide (*ATP7A*), biogenesis of lysosomal organelles complex-1, subunit 5 (*BLOC1S5*), cannabinoid receptor 1 (*CNR1*), dickkopf 1 (*DKK1*), dystrobrevin binding protein 1 (*DTNBP1*), melanocortin 1 receptor (*MC1R*), microphthalmia-associated transcription factor (*MITF*), melanophilin (*MLPH*), myosin VA (*MYO5A*), paired box 3 (*PAX3*), biogenesis of lysosomal organelles complex-1, subunit 6, pallidin (*PLDN*), premelanosome protein (*PMEL*), RAB27A, member RAS oncogene family (*RAB27A*), guanine nucleotide binding protein (G protein), beta polypeptide 2-like 1 (*RACK1*) and SRY-box 10 (*SOX10*) were extracted for our analysis. *ATP7A, DTNBP1, BLOC1S5, PLDN* and *PMEL* are involved in melanosomal biogenesis (6, 10). *RAB27A, MYO5A* and *MLPH* are genes encoding for transfer proteins, relevant for melanosomal transport within the melanocyte (6, 10). *MC1R, MITF, PAX3* and *SOX10* are involved in important melanocyte signaling pathways (6, 10). *DKK1, RACK1* and *CNR1* are associated with skin pigmentation *via* other mechanisms (6, 10-12).

Clinical data. Clinical data were extracted from the LURIC database, as described previously (2). For 2,974 out of the 3,316 patients, both genotypic data and 25(OH)D serum concentrations were available, and these individuals were included in our analysis. The remaining 342 patients were excluded. For some individuals, single SNP data were missing. These persons were also excluded from the analysis of the respective SNPs.

Statistical methods. Statistical methods have been described in detail previously (2). In short, we calculated for each genotype (for example AA, AT and TT) of the 244 SNPs the mean, median and standard deviation of 25(OH)D serum concentration. We used the Kolmogorov-Smirnov-Test to analyze whether the distribution of the 25(OH)D serum concentration in our cohort was different from equality. Because of non-equality, we chose the non-parametric Kruskal-Wallis analysis of variance (ANOVA)-Test (SPSS 20, IBM Co., Armonk, US) to access as to whether there is a difference within the medians of 25(OH)D values between the 3 possible genotype combinations of each SNP in the total cohort (2).

Considering a two-sided significance level of 0.05 for this exploratory analysis, we identified 11 SNPs in this first screening approach. Next, we used an allelic test to investigate which one of the allelic variants (*e.g.* C or G) was associated with significant difference in the 25(OH)D concentration. Because there were no data available whether these SNPs are dominant, recessive or codominant, we tested them in each allelic model: the dominant, the recessive and the multiplicative model (for codominant). For statistical comparisons, the Kruskal-Wallis or Mann-Whitney *U*-test was used, respectively. We had to exclude 3 SNPs (rs10521358, rs2227291 and rs17139617) from analysis, because they were not in the Hardy-Weinberg-Equilibrium (HWE) (2).

To account for multiple testing in this allelic approach, we used the false discovery rate (FDR) according to the procedure of Benjamini-Hochberg (2).

Moreover, we used a multiple linear regression model adjusted to BMI, sex, month of blood sample and age-group and estimated the impact of individuals SNPs on 25(OH)D concentration. We also checked with Kaplan-Meyer analysis and Cox Regression as to whether one of the identified SNPs has an effect on survival.

Because of existing correlations between 25(OH)D and parathormone levels (PHT) (13, 14), the 11 SNPs identified in our first approach were additionally tested with Kruskal-Wallis analysis of variance (ANOVA) (SPSS 20, IBM Co., Armonk, NY, USA) to investigate differences in median values of parathormone for different genotypes.

Results

In a cohort of the LURIC study, we tested the hypothesis whether variants of fifteen genes (SNPs) that are of high importance for skin pigmentation, namely *ATP7A, DTNBP1, BLOC1S5, PLDN, PMEL* (involved in melanosomal biogenesis); *RAB27A, MYO5A, MLPH* (encoding transfer proteins relevant for melanosomal transport within melanocytes); *MC1R, MITF, PAX3, SOX10* (involved in melanocyte signaling pathways), *DKK1, RACK1, CNR1* (associated with skin pigmentation *via* other mechanisms) are genetic determinants of 25(OH)D serum concentration.

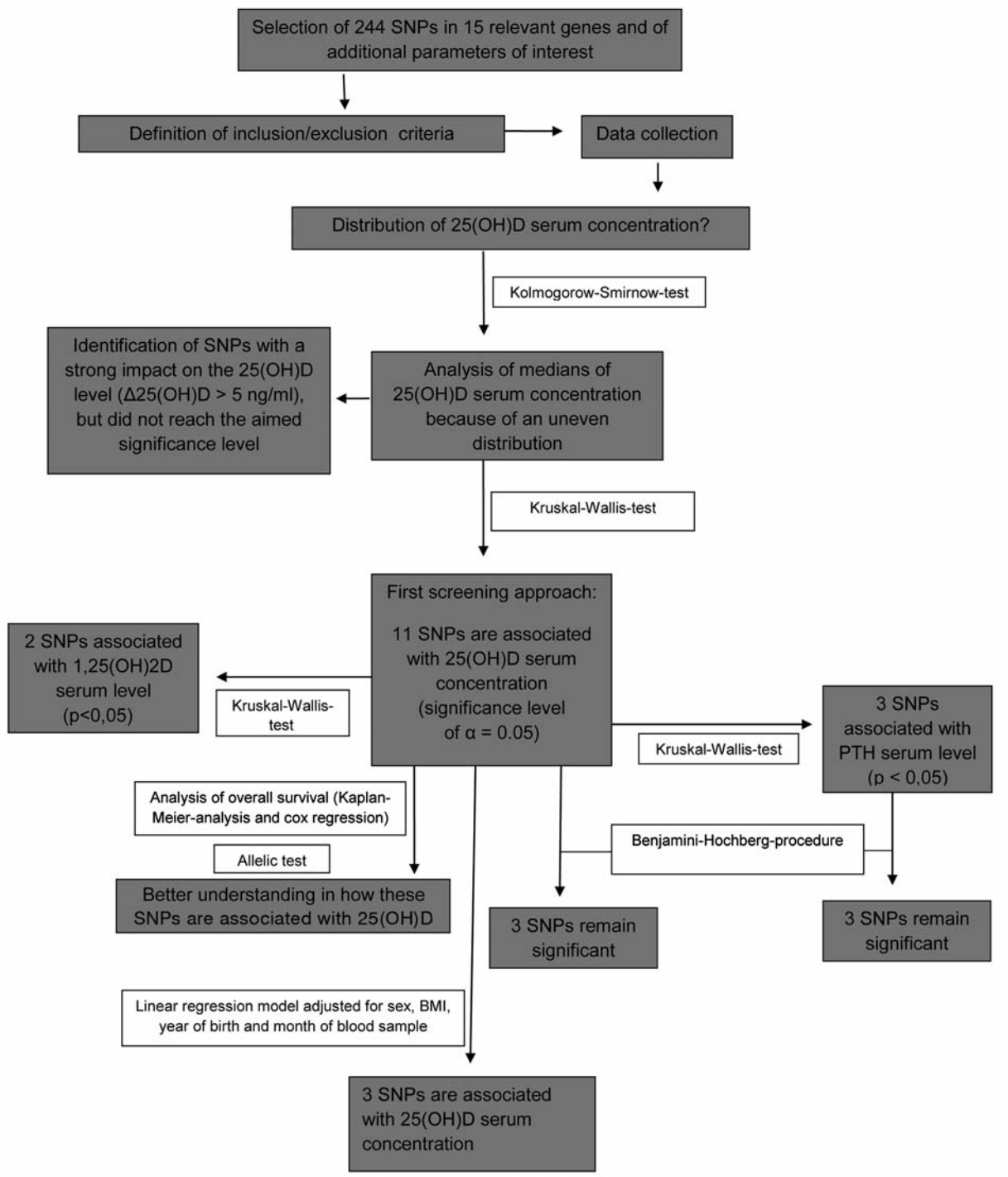

Figure 1. *Algorithm of our study design to assess (n=2974) whether variants (n=244) of genes (n=15) involved in skin pigmentation are associated with vitamin D status in a cohort of caucasian individuals.*

We included 2,974 patients (29.83% females, 70.17% males) with a mean 25(OH)D serum concentration of 17.3 ng/ml and a median of 15.5 ng/ml and excluded patients with missing data (2).

In our first screening approach on a significance level of α=0.05, 11 SNPs (25(OH)D medians from highest to lowest) located in 6 different genes were associated with low or high serum 25(OH)D levels, compared with the total cohort

(median=15.5 ng/ml) or across the allelic variants (Table II). The significance of these 11 SNPs was then tested by applying a well-accepted correction procedure for multiple analyses (false discovery rate, FDR, Benjamini-Hochberg procedure), to minimize the probability of a statistical type I error (2). After FDR 3 out of these 11 SNPs were still within the significance level, namely rs10521358 (ATP7A) p=0.000025, rs2227291 (*ATP7A*) p=0.000047 and rs17139617 (*ATP7A*) p=0.000096 (Table II).

Next, we used allelic tests to investigate which one of the allelic variants (*e.g.* C or G) was associated with a significant difference in the 25(OH)D concentration. Because there were no data available whether these SNPs are dominant, recessive or codominant, we tested them in each allelic model (Table III): The dominant model, the recessive model and the multiplicative model (for codominant). We had to exclude 3 SNPs (rs10521358, rs2227291 and rs17139617) because they were not in the Hardy-Weinberg-Equilibrium (HWE) (2). In the dominant model of the allelic test these SNPs showed p-values below 0.05: rs7565264 (*MLPH*), rs7569427 (*MLPH*), rs2292881 (*MLPH*), rs12469812 (*MLPH*), rs9328451 (*BLOC1S5*), rs10932949 (*PAX3*). The codominant model revealed significant p-values for rs7565264 (*MLPH*), rs9328451 (*BLOC1S5*), rs2292881 (*MLPH*), rs7569427 (*MLPH*), rs12469812 (*MLPH*) while in the recessive model the SNPs rs2069408 (*PMEL*) and rs6454677 (*CNR1*) reached significant p-values.

In the linear regression model adjusted for sex, body mass index (BMI), year of birth and month of blood sample the following SNPs showed a significant association with 25(OH)D: rs7565264 (*MLPH*), rs10932949 (*PAX3*), rs9328451 (*BLOC1S5*).

Then, we tested the combined impact of SNPs on the variation of 25(OH)D serum level. The coefficient of determination (R^2) for the 11 SNPs identified in our first approach was 1.6% and for the 3 SNPs after FDR 0.3%. We also identified a group of SNPs, that were associated with high Δ25(OH)D values, but did not reach the aimed significance level. These SNPs are shown in Table IV. We also investigated the association of the 11 SNPs identified in our first approach with survival. In Cox Regression we identified rs2292881 (*MLPH*) for having a significant advantage in overall survival (p=0.040). Kaplan-Meier analysis did not show a significant impact of individual SNPs on overall survival.

Furthermore, we tested whether the 11 SNPs identified in our first approach also show an association with $1,25(OH)_2D$ (mean=34.91 ng/l, median=33.2 ng/l) and/or parathormone (PTH) (mean=33.88 pg/ml, median=29.00 pg/ml) serum concentration. The SNPs rs17139617 (p=0.006) and rs2227291 (p=0.014) (both in *ATP7A*) showed an association with $1,25(OH)_2D$ serum concentration, the SNPs rs10521358 (p=0.008561), rs2227291 (p=0.000209) and rs17139617 (p=0.000075) (all in *ATP7A*) showed an association with PTH serum concentration (Table V). After FDR the associations between these SNPs and PTH remained significant,

Discussion

The pigmentation pathway of the skin represents a complex and strictly regulated network of various hormones, corresponding receptors and other factors (Figure 2), being at present still not completely understood (6, 10-12). One important mechanism is melanosomal biogenesis, that includes the melanosome's task to synthesize eumelanins and pheomelanins, and that involves various enzymes and related factors, *e.g.* tyrosinase, tyrosinase-related protein 1 and dopachrome tautomerase (6). Other hallmarks that regulate and determine a person's individual skin pigmentation are melanosomal transport and transfer, to which microtubules, dynein, kinesin, actin filaments, Rabd27a, melanophilin, myosin Va and Slp2-a contribute (6). However, many other factors including various transcription factors such as *MITF, PAX3* or *SOX10* or the melanocortin-1 receptor (*MC1R*) are involved in skin pigmentation (6), whose variability is of great importance for human evolution (3). It has been hypothesized that, in order to guarantee a sufficient amount of cutaneous vitamin D synthesis, humans had to brighten their dark skin pigmentation before they could move north from the equator. Although some progress has been made in recent years, explaining the diversity of skin pigmentation is still one of the major unanswered questions of human genetics (2). Only in the last few years has it been possible to address this unresolved question by applying approaches of population genetics. Based on preliminary investigations in animals and on earlier studies in humans with Mendelian disorders, GWAS have now identified many genes that contribute to the variability of skin pigmentation (6, 15). On the basis of these results we had chosen SNPs in 29 different genes with relevance for key mechanisms of skin pigmentation, (including melanin synthesis, tyrosine transport and melanogenic signaling) for our previous pilot study that tested the hypothesis whether variants of genes involved in pigmentation pathways are predictive of serum 25(OH)D levels (2). In that study, we identified the association of several genes related to skin pigmentation, including *EXOC2, TYR, TYRP1* and *DCT* with vitamin D status (2). Due to these scientifically highly interesting results, we decided to extend our analysis, focusing on other hallmarks of the pigmentation pathway. For our present study, we chose 15 additional genes with great relevance for different aspects of skin pigmentation, including melanosomal biogenesis (*ATP7A, PMEL, PLDN*), melanosomal transport (*RAB27A, MLPH*), or various melanocyte signaling pathways (*MITF, PAX3*). However, it should be noted that these genes exert many other functions unrelated to the pigmentation pathway. For example, the copper ATPase *ATP7A*, located on the X chromosome (16),

Table II. *Association between SNPs with relevance for skin pigmentation and vitamin D status: results of our first screening approach.*

Nearest gene	SNP	*p*-Value		25(OH)D concentration (ng/ml)	ΔMedian (M(total) - M(SNP)) (ng/ml)
ATP7A	rs10521358	0.000025	C C (n=489)	16.2	–0.7
			C G (n=307)	12.8	2.7
			G G (n=2162)	15.7	–0.2
			Total (n=2958)	15.5	
ATP7A	rs2227291	0.000047	C C (n=505)	16.1	–0.6
			C G (n=298)	12.8	2.7
			G G (n=2133)	15.9	–0.4
			Total (n=2936)	15.5	
ATP7A	rs17139617	0.000096	A A (n=505)	16.1	–0.6
			A C (n=308)	12.85	2.65
			C C (n=2158)	15.7	–0.2
			Total (n=2971)	15.5	
MLPH	rs7565264	0.000992	T T (n=22)	13.1	2.4
			T C (n=402)	17.7	–2.2
			C C (n=2543)	15.2	0.3
			Total (n=2967)	15.5	
PAX3	rs10932949	0.004	A A (n=116)	13.9	1.6
			A G (n=933)	16.4	–0.9
			G G (n=1918)	15.2	0.3
			Total (n=2967)	15.5	
PMEL	rs2069408	0.015	G G (n=318)	17.05	–1.55
			G A (n=1313)	15.0	0.5
			A A (n=1343)	15.6	–0.1
			Total (n=2974)	15.5	
MLPH	rs7569427	0.026	G G (n=21)	15.3	0.2
			G A (n=464)	16.95	–1.45
			A A (n=2489)	15.3	0.2
			Total (n=2974)	15.5	
BLOC1S5	rs9328451	0.028	T T (n=87)	14.6	0.9
			T C (n=867)	14.7	0.8
			C C (n=2017)	16.0	–0.5
			Total (n=2971)	15.5	
MLPH	rs12469812	0.03	T T (n=17)	12.5	3
			T C (n=408)	17.2	–1.7
			C C (n=2548)	15.3	0.2
			Total (n=2973)	15.5	
MLPH	rs2292881	0.041	T T (n=27)	15.4	0.1
			T C (n=441)	16.8	–1.3
			C C (n=2506)	15.3	0.2
			Total ((n=2974)	15.5	
CNR1	rs6454677	0.046	T T (n=22)	22.9	–7.4
			T A (n=518)	15.45	0.05
			A A (n=2422)	15.5	0
			Total (n=2962)	15.5	

Our first screening approach using Kruskal-Wallis one-way analysis of variance (ANOVA)-Test or Mann-Whitney *U*-Test on a significance level of α=0.05 identified 11 SNPs that were associated with 25(OH)D serum concentration.

plays an important role in intracellular copper homeostasis both by pumping copper into the Golgi compartments of cells, and by removing excess copper *via* relocation to the plasma membrane. Mutations in ATP7A may lead either to Menkes Disease, a milder variant called occipital horn syndrome, or a phenotypic variant that involves a later-onset isolated X-linked distal motor neuropathy (DMN) (16).

In the screening approach of our present work on a significance level of α=0.05, we identified for 11 SNPs, for the first time, associations with low or high 25(OH)D serum concentrations. Interestingly, one SNP (rs6454677, *CNR1*, p=0.046) was associated with a relatively strong effect on vitamin D status (Δ25(OH)D=7.4 ng/ml), while the effects of the remaining 10 SNPs related to *ATP7A* (rs10521358,

Table III. *Association between SNPs with relevance for skin pigmentation and vitamin D status: results of the allelic test.*

Nearest gene	SNP	Multiplicative model	Dominant model	Recessive model
MLPH	rs7565264	0.016	0.006	0.138
PAX3	rs10932949	0.248	0.042	0.069
PMEL	rs2069408	0.175	0.933	0.005
MLPH	rs7569427	0.026	0.012	0.595
BLOC1S5	rs9328451	0.02	0.027	0.836
MLPH	rs12469812	0.036	0.021	0.275
MLPH	rs2292881	0.022	0.014	0.984
CNR1	rs6454677	0.229	0.454	0.029

Results of the allelic test to investigate which one of the allelic variants (*e.g.* C or G) of the 11 SNPs identified in our screening approach was associated with significant differences in the 25(OH)D concentration are shown. Because there were no data available on whether these SNPs are dominant, recessive or co-dominant, we tested them in each allelic model: the dominant, the recessive and the multiplicative model (for co-dominant). We had to exclude 3 SNPs (rs10521358, rs2227291 and rs17139617) because they were not in the Hardy-Weinberg-Equilibrium (HWE).

Table IV. *SNPs with relevance for skin pigmentation and vitamin D status: SNPs with strong impact on 25(OH)D level*

Nearest gene	SNP	ΔMedian (M(total) - M(SNP))	SNPs (n)
MLPH	rs6705903	–14.7 ng/ml	1
DTNBP1	rs2056943	–7.5 ng/ml	4
CNR1	rs2057276	–7.4 ng/ml	1
SOX10	rs3026645	–7.1 ng/ml	7
MYO5A	rs12899847	–7 ng/ml	13
MYO5A	rs12592429	–7 ng/ml	9
MYO5A	rs12915892	–7 ng/ml	9
CNR1	rs9450901	–6.1 ng/ml	23
MITF	rs10510992	–5.6 ng/ml	5

We identified a group of SNPs located in genes MLPH (rs6705903), DTNBP1 (rs2056943), CN1R (rs2057276, rs9450901), SOX10 (rs3026645), MYO5A (rs12899847, rs12592429, rs12915892) and MITF (rs10510992), which had a strong impact on 25(OH)D level (Δ25(OH)D >5 ng/ml), but did not reach the aimed significance level.

rs2227291, rs17139617), *MLPH* (rs7565264, rs7569427, rs12469812, rs2292881), *PAX3* (rs10932949), *PMEL* (rs2069408), and *BLOC1S5* (rs9328451), were less pronounced (Δ25(OH)D<5 ng/ml). The relevance of these results is underlined by the observation that rs6454677 showed a higher Δ25(OH)D compared to each of the SNPs that were identified on a significance level of α=0.05 (n=46) in our previous pilot study, in which we investigated a total of 960 genetic variants.

Next, we used allelic tests to investigate which one of the allelic variants (*e.g.* C or G) was associated with significant differences in 25(OH)D serum concentration. Because no data were available on whether individual SNPs are dominant, recessive or co-dominant, we tested them in each allelic model (dominant model, recessive model and multiplicative model (for codominant)). We had to exclude 3 SNPs (rs10521358, rs2227291 and rs17139617) because they were not in the Hardy-Weinberg-Equilibrium (HWE) (2). Using allelic tests, we confirmed the significance of the remaining 8 SNPs (rs7565264 (*MLPH*), rs10932949 (*PAX3*), rs2069408 (*PMEL*), rs7569427 (*MLPH*), rs9328451 (*BLOC1S5*), rs12469812 (*MLPH*), rs2292881 (*MLPH*), rs6454677 (*CNR1*)).

When we re-tested the significance of the SNPs identified in our first approach using a well-accepted correction procedure for multiple analyses (false discovery rate, FDR, Benjamini-Hochberg procedure), to minimize the probability of a statistical type I error, 3 out of these, 11 SNPs were still within the aimed significance level: rs10521358 (*ATP7A*) p=0.000025, rs2227291 (*ATP7A*) p=0.000047 and rs17139617 (*ATP7A*) p=0.000096 (Table II).

Table V. *SNPs with relevance for skin pigmentation and vitamin D status: rs2227291 and rs17139617 (both in ATP7A) are associated with 1,25(OH)$_2$D serum concentration, rs10521358, rs2227291 and rs17139617 (all in ATP7A) are associated with PTH serum concentration.*

Parameter	Nearest gene	SNP	p-Value
1,25(OH)2D serum concentration	ATP7A	rs17139617	0.006
	ATP7A	rs2227291	0.014
PTH serum concentration	ATP7A	rs17139617	0.000075
	ATP7A	rs2227291	0.000209
	ATP7A	rs10521358	0.008561

Out of the 11 SNPs identified in our first approach, rs2227291 and rs17139617 (both in ATP7A) showed an association with 1,25(OH)^{2}D serum concentration, while SNPs rs10521358, rs2227291 and rs17139617 (all in ATP7A) showed an association with PTH serum concentration. After FDR the associations between these SNPs and PTH remained significant.

It is well-known that many individual factors such as age, sex, body mass index (BMI), and season influence 25(OH)D serum concentration (1). Our linear regression analysis adjusting for these factors showed for three SNPs (rs7565264 (*MLPH*), rs10932949 (*PAX3*), and rs9328451 (*BLOC1S5*)) a significant association with 25(OH)D serum concentration.

When we assessed the impact of these associations for vitamin D status, the 11 SNPs identified in our first screening approach and the 3 SNPs remaining after FDR reached a coefficient of determination (R^2) of 1.6% and 0.3%, respectively. In conclusion, up to 1.6% of the variation in 25(OH)D concentration can be explained by these 11 SNPs. In 2010, a genome-wide association study

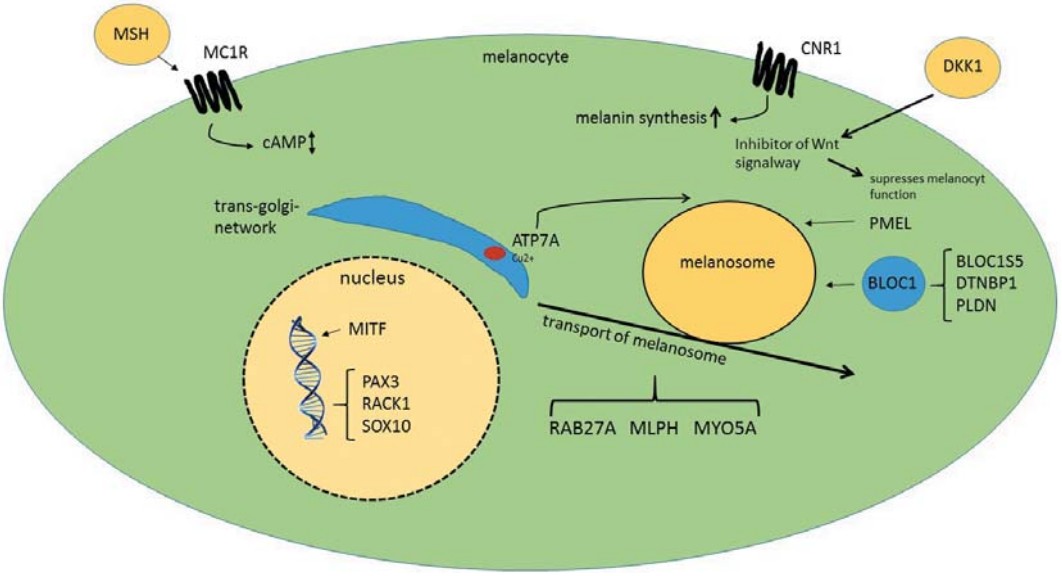

Figure 2. *Illustration of the function of our analyzed genes and how they are involved in the pigmentation pathway. PAX3 and SOX10 are transcription factors in the melanocyte. RACK1 is a regulator to the signal transduction in the melanocyte. PMEL, BLOC1S5, DTNBP1 and PLDN are genes which are involved in the genesis of the melanosome, which is important for the pigmentation process. DKK1, MC1R, MITF, ATP7A, CN1R RAB27A, MLPH and MYO5A encode for proteins in the membrane or cytoplasm of the melanocyte and which are involved in signalling pathways of skin pigmentation. We did not show the important tyrosinase pathway because this was analyzed in earlier studies. MSH: Melanocyte-stimulating hormone, MC1R: melanocortin 1 receptor, cAMP: 3',5'-cyclic AMP, CN1R: cannabinoid receptor 1, DKK1: Dickkopf WNT signaling pathway inhibitor 1, PMEL: premelanosome protein, BLOC1S5: biogenesis of lysosomal organelles complex-1 subunit 5, DTNBP1: dystrobrevin binding protein 1, PLDN: biogenesis of lysosomal organelles complex-1subunit 6, BLOC1: biogenesis of lysosomal organelles complex-1, ATP7A: Cu^{++} transporting alpha polypeptide, MITF: microphtalmia-associated transcription factor, PAX3: paired box 3, RACK1: guanine nucleotide binding protein, SOX10: SRY (sex determining region Y)-box 10, RAB27A: member RAS oncogene family, MLPH: melanophilin, MYO5A: Myosin VA (heavy chain 12, myoxin).*

has identified genetic variants at three loci, namely 4p12 (rs2282679 in GC), 11q12 (rs12785878 near *DHCR7*), and 11p15 (rs10741657 near *CYP2R1*) as determinants of vitamin D insufficiency [4]. In this study, SNPs at the three confirmed loci explained up to 1%-4% of the variation in 25(OH)D concentrations (4). It has to be noted that, most likely because of the heterogeneous cohort and conservative data analysis that may have led to a statistical type II error (*i.e.* overlooking of associations), genes related to skin pigmentations were not identified by this GWAS. Our study design to examine a homogeneous cohort of Caucasians from the area of Ludwigshafen has weaknesses and strengths. One strength is the homogeneousity of our cohort, with many important factors that influence the vitamin D status (including metereological or geographical conditions and lifestyle) being common in all individuals. The analysis of other populations would have been gone far beyond the aim of our study.

Weaknesses of our study are the facts that no information on skin type was available, that vitamin D status was only assessed by a single measurement, and that 25(OH)D measuremens were not confirmed by mass spectrometry.

Additional limitations include the possibility, that the relatively low numbers of relevant genetic variants (*i.e.* a low minor allele frequency) may have led to overlooking of associations (statistical type II error). Therefore we identified another group of SNPs located in genes MLPH (rs6705903), DTNBP1 (rs2056943), CN1R (rs2057276, rs9450901), SOX10 (rs3026645), MYO5A (rs12899847, rs12592429, rs12915892) and MITF (rs10510992), which had a strong impact on the 25(OH)D level (Δ25(OH)D > 5 ng/ml), but did not reach the aimed significance level (Table IV).

An increasing body of evidence now convincingly demonstrates the association of vitamin D deficiency with a worse outcome and an increased incidence of many diseases, including various types of cancer, infectious, autoimmune and cardiovascular diseases (1, 3). When we analyzed the impact of the 11 SNPs identified in our first approach on overall survival one SNP (rs2292881, *MLPH*) reached the aimed significance in Cox Regression and was close to it (p=0.055) in the Kaplan-Meier analysis. However, due to its relatively weak impact on vitamin D status (Δ25(OH)D=1.3 ng/ml), it is very unlikely that the association of this SNP with overall survival is caused by effects on 25(OH)D serum concentration.

Last but not least we demonstrated that, out of the 11 SNPs identified in our first approach, rs2227291 and rs17139617 (both in *ATP7A*) showed an association with $1,25(OH)_2D$ serum concentration, while SNPs rs10521358, rs2227291 and rs17139617 (all in *ATP7A*) showed an association with PTH serum concentration. After FDR the associations between these SNPs and PTH remained significant. The associations of rs10521358, rs2227291 and rs17139617 (all in *ATP7A*) with PTH serum concentration confirmed our findings, for they are well in line with the fact that blood concentrations of PTH and 25(OH)D correlate (13, 14). $1,25(OH)_2D$ and 25(OH) D serum concentrations may correlate in vitamin D-deficient individuals as well (13, 14).

Skin type and skin pigmentation are individual factors which predispose for a person's vitamin D status (1). In summary, our study supports this concept, through identifying that several SNPs related to pigmentation are new genetic determinants of 25(OH)D serum concentration in the Caucasian population.

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Received January 31, 2016
Revised February 16, 2016
Accepted February 18, 2016