CALL FOR PAPERS | Technology Development for Physiological Genomics

Metabolomic profiles indicate distinct physiological pathways affected by two loci with major divergent effect on *Bos taurus* growth and lipid deposition

Rosemarie Weikard,1 Elisabeth Altmaier,2 Karsten Suhre,2 Klaus M. Weinberger,3 Harald M. Hammon,4 Elke Albrecht,5 Kouji Setoguchi,6 Akiko Takasuga,7 and Christa Kühn1

1Research Unit Molecular Biology, Leibniz Institute for Farm Animal Biology (FBN), Dummerstorf; 2Institute of Bioinformatics and Systems Biology, Helmholtz Zentrum München, Neuherberg, Germany; 3Biocrates Life Sciences Aktiengesellschaft, Innsbruck, Austria; Research Units 4Nutritional Physiology “Oskar Kellner” and 5Muscle Biology and Growth, Leibniz Institute for Farm Animal Biology (FBN), Dummerstorf, Germany; 6Cattle Breeding Development Institute of Kagoshima Prefecture, Osumi, Saga, Kagoshima; and 7Shirikawa Institute of Animal Genetics, Japan Livestock Technology Association, Odakura, Nishigo, Fukushima, Japan

Submitted 18 June 2010; accepted in final form 14 July 2010

Weikard R, Altmaier E, Suhre K, Weinberger KM, Hammon HM, Albrecht E, Setoguchi K, Takasuga A, Kühn C. Metabolomic profiles indicate distinct physiological pathways affected by two loci with major divergent effect on *Bos taurus* growth and lipid deposition. *Physiol Genomics* 42A: 79 –88, 2010. First published July 20, 2010; doi:10.1152/physiolgenomics.00120.2010.—Identifying trait-associated genetic variation offers new prospects to reveal novel physiological pathways modulating complex traits. Taking advantage of a unique animal model, we identified the I442M mutation in the non-SMC condensin I complex, subunit G (*NCAPG*) gene and the Q204X mutation in the growth differentiation factor 8 (*GDF8*) gene as substantial modulators of pre- and/or postnatal growth in cattle. In a combined metabolomic and genotype association approach, which is the first respective study in livestock, we surveyed the specific physiological background of the effects of both loci on body-mass gain and lipid deposition. Our data provided confirming evidence from two historically and geographically distant cattle populations that the onset of puberty is the key interval of divergent growth. The locus-specific metabolic patterns obtained from monitoring 201 plasma metabolites at puberty mirror the particular *NCAPG* I442M and *GDF8* Q204X effects and represent biosignatures of divergent physiological pathways potentially modulating effects on proportional and disproportional growth, respectively. While the *NCAPG* I442M mutation affected the arginine metabolism, the Q204X allele in the *GDF8* gene predominantly raised the carnitine level and had concordant effects on glycerophosphatidylcholines and sphingomyelins. Our study provides a conclusive link between the well-described growth-regulating functions of arginine metabolism and the previously unknown specific physiological role of the *NCAPG* protein in mammalian metabolism. Owing to the confirmed effect of the *NCAPG/LCORL* locus on human height in genome-wide association studies, the results obtained for bovine *NCAPG* might add valuable, comparative information on the physiological background of genetically determined divergent mammalian growth.

Non-SMC condensin I complex, subunit G gene; arginine; disproportional growth; carnitine; genetic association

---

THE GENETIC AND PHYSIOLOGICAL mechanisms and interactions underlying the genetically determined variation in mammalian pre- and postnatal growth and lipid metabolism are still only partially understood. Identifying trait-associated genetic variation offers new prospects to reveal novel physiological pathways modulating such complex traits. Large animal models as supplied by livestock populations can provide substantial information about the genetic and physiological mechanisms underlying the genetically determined variation of complex traits, especially when targeting pre- and postnatal development: Livestock species offer uniparous models, a high phenotypic variability for complex traits in outbred populations due to long-term selective breeding, suitable sample sizes, and high-quality genome sequences (4).

In a previous study, we identified the nonsynonymous mutation I442M (c.1326T>G) in the bovine nonstructural maintenance of chromosomes (SMC) condensin I complex, subunit G (*NCAPG*) gene on *Bos taurus* chromosome 6 (BTA6) to be strongly associated with prenatal growth in cattle (18). Further data on the bovine *NCAPG* gene (45), as well as comparative information from the human and mouse genomes (23, 27, 47, 54), suggested that the *NCAPG* locus might also be associated with postnatal growth and lipid deposition. Until now, data on the physiological function of the *NCAPG* gene in mammals have been scarce. *NCAPG* has a catalytic function in the mammalian condensin I complex and is important during mitotic cell division (15). Owing to its interaction with the DNA methyltransferase 3β, *NCAPG* links the regulation of DNA methylation with mitotic chromosome condensation in mammalian cells (20). However, a detailed analysis of the role of *NCAPG* in mammalian physiological pathways has yet to be undertaken.

Recently, progress in mass spectrometry has enabled extensive targeted metabolomic studies on body fluids providing a direct readout of biological processes (24). The resulting comprehensive analysis of specific targeted plasma metabolite concentrations can reveal details on the physiological background of genetically determined differences in physiological processes and disclose new regulatory pathways for complex traits as demonstrated in recent studies in human and mice (16, 24). For large animal models, e.g., ruminants, comprehensive metabolomic plasma analyses are lacking despite their potential to improve our understanding of genetically determined variation of ruminant physiology and comparative physiology of mammals.
Our experimental population facilitated discriminating the specific effects of the NCAPG I442M mutation from unspecific growth-related effects due to the parallel segregation of the NCAPG I442M locus and the disrupting Q402X mutation in the myostatin encoding growth differentiation factor 8 (GDF8). As a consequence, the effects of both loci could be monitored on an identical genetic background. Myostatin is a well-characterized inhibitor of the myoblast terminal differentiation (29, 41) and myogenic cell proliferation (50). The GDF8 gene is well confirmed as a major locus affecting growth and lipid deposition in mammals. In cattle, a variety of mutations are described (17). However, most association studies, e.g., Refs. 22, 35, focused on carriers of the c.819_829del 11 bp deletion or the F94L (c.283C>A) mutation. In the current study, we combined meticulous phenotypic monitoring and genetic association analyses with the new method of targeted metabolomic profiling to elucidate the physiological background of the divergent genetic effects of the NCAPG I442M and GDF8 Q204X mutations on growth and lipid deposition. Our metabolomic data provided discriminating biosignatures for two distinct loci, NCAPG I442M and GDF8 Q204X, both of which are associated with divergent postnatal body-mass gain and lipid deposition. Interestingly, the amino acid arginine and its metabolite symmetric dimethylarginine showed a significant association with the NCAPG I442M mutation. Arginine is frequently proposed as a food additive due to the important regulatory role of arginine and its metabolites in pre- and postnatal growth in many species (58).

MATERIALS AND METHODS

Animals. Our study comprised 156 male individuals from an F2 resource population (28) generated from the founder breeds Charolais and German Holstein. All F1 and F2 animals of the resource population were generated by multiple ovulation and embryo transfer to dissect any potential systematic effect of maternal alleles on intrauterine development from the specific effect of the fetal alleles. Embryos were transferred randomly to virgin heifers kept in the experimental herd of the FBN Dummerstorf according to a standard protocol. The calves were weaned from their mothers immediately after birth. Until day 121 post natum, the calves were fed a milk replacer diet according to requirements, which was gradually replaced by ad libitum access to hay and concentrates. Subsequently, the individuals were kept in a tie-stall barn on a daily semi-ad libitum feed ration, which was composed of chaffed hay and a concentrate (RM 2007; Vollkraft Mischfutterwerke, Rendsburg, Germany) with a hay-to-concentrate ratio of 1:3 and an energy content of 12.7 MJ ME/kg dry matter. The ration provided for a maximal average daily weight gain, while still being compatible with ruminant requirements. All experimental procedures were carried out according to the German animal care guidelines and were approved and supervised by the relevant authorities of the State Mecklenburg-Vorpommern, Germany.

For a confirmation study of the effects of the NCAPG I442M mutation on postnatal growth, a Japanese black (Wagyu) population used for the official progeny test was investigated. A total of 792 steers were tested under standardized environmental conditions determined for progeny testing by the Japan Wagyu Register Association (36). The individuals entered the test station at 271 days of age (average age, ± 12 days SD) and were fed an ad libitum diet of concentrates (total digestible nutrients 73.0%, crude protein 12.0%) and roughage (timothy) during the test period of 364 days.

Monitoring of growth and lipid deposition. The average daily weight gain for all individuals from the experimental resource population was obtained from monthly weight measurements and calculated for six intervals from birth to day 547 (Supplemental Table S1),1 when all individuals were slaughtered at the same age. After death, a detailed dissection of the body (Supplemental Table S2) was performed to monitor the differences of the weight of the different body compartments as well as the total amount and the specific depots of body fat deposition (38).

For the confirmation population from the Japanese Wagyu breed, the average daily gain was recorded in 2-mo intervals from day 271 to day 607 (Supplemental Table S1).

Genetic markers. The NCAPG I442M locus (NCAPG c.1326T>G) was genotyped in all P0, F1, and F2 individuals of the resource population, as described by Eberlein et al. (18). For genotyping all P0, P1, and F2 individuals at the GDF8 Q204X (GDF8 c.611C>T) mutation, a Tetra-ARMS assay was applied with the following primers (5'-3') and PCR conditions: embracing forward primer: AGACT-CATCACAACCCCATGAAAG (0.2 μM), embracing reverse primer: TGAAGTACAGGGCTACACTGG (0.2 μM), specific inner forward primer: ACTCAGGCACTGGTATTTGGT (0.2 μM), specific inner reverse primer: ACTGTCTTCACATCATAATCTCTG (1 μM) at 60°C annealing temperature. All genotypes were meticulously checked for inconsistencies according to a previously described protocol (18).

Metabolomic analyses. Because the initial association analyses had suggested that the most divergent growth was observed between 180 and 270 days after birth, a targeted metabolome scan on plasma samples from 156 individuals at the age of 240 days was performed. To avoid any potential effects on plasma metabolite concentration due to differences in circadian rhythms or feeding, all blood samples were drawn from the left jugular vein into K-EDTA-containing tubes at 7:30 AM with a prior fasting time for the individuals of 12 h. Blood samples were immediately stored on ice and transferred to the laboratory, where plasma was obtained by centrifugation within 30 min after blood sampling. The plasma was stored at −80°C until further analysis.

For the targeted metabolomic analysis, essentially, plasma metabolites were determined by electrospray ionization tandem mass spectrometry with the Biocrates AbsoluteIDQ targeted metabolomics technology analogously to Refs. 3, 21, 24. Briefly, a targeted profiling scheme is applied to quantitatively screen for known small-molecule metabolites using multiple reaction monitoring, neutral loss, and precursor-ion scans. Appropriate internal standards served as reference for quantification of the metabolites in the plasma samples. Details of the experimental metabolomics measurement techniques are described in the patent US 2007/0004044 (accessible online at http://www.freepatentsonline.com/20070004044.html). A further summary of the method can be found in Refs. 55, 56, and a comprehensive overview of the field and the related technologies is given by Winck (57). Because initial data and results on GDF8 Q204X in the literature (2) had suggested effects of both genes, NCAPG and GDF8, on lipid deposition, the investigation of plasma metabolites focused on indicators of lipid metabolism: free carnitine (C0), acylcarnitines (Cx:y), hydroxylacylcarnitines [C(OH)x:y], dicarboxyacylcarnitines (Cx:yDC), acylglycerophosphatidylcholines (lysoPCx:y), diacylglycerophosphatidylcholines (PCaeCx:y), diacylglycerophosphatidylcholines (PCaaCx:y), acetylglycyrophosphatidylcholines (PCaeCx:y), sphingomyelins (SMx:y), N-hydroxydicarboxyloylphosphatidylcholines [SM(OH)COx:y], and N-hydroxyacycloxyphosphatidylcholines [SM(OH)acyCx:y]. The lipid side chain formation is abbreviated as Cx:y, where x summarizes the number of carbons, and y indicates the number of double bonds in the side chains. The set of lipid metabolites was complemented by amino acids, sugars (with Hr corresponding to n-hexose, dh to deoxyhexose, UA to uronic acid, HNAC to N-acetylgulosamine, P to Pentose, NANA to N-acetylcarnaminic acid), and biogenic amines. A full list of all tested metabolites, as well as their acronyms, mean

1 The online version of this article contains supplemental material.
levels, and standard deviations (in μM), is provided in Supplemental Table S3.

Statistical analyses. Association linkage disequilibrium (LD) studies in the experimental resource population were performed essentially as described by Eberlein et al. 18 to evaluate, if the target mutations NCAPG I442M and GDF8 Q204X were associated with additive effects on growth, lipid deposition, or metabolic profile. The following model as implemented in the LD association analysis in Qxpak (37) was applied: yi = s0 + λgk + ui + εik, where γi is the record of individual i, s0 is the fixed effect of slaughter year d, λ is an indicator variable, which is 1, 0, or −1 depending on the genotype of individual i at locus NCAPG I442M or GDF8 Q204X, gk is the additive allelic effect of allele k at locus NCAPG I442M, ui is an indicator variable, which is 1, 0, or −1 depending on the genotype of individual i at locus NCAPG I442M or GDF8 Q204X, εik is the additive allelic effect of allele j at locus GDF8 Q204X, ui is the infinitesimal genetic effect of individual i, and εik is the residual. For analyzing metabolites, we added a fixed effect wk of the analysis plate g to the model.

To evaluate joined effects of NCAPG I442M and GDF8 Q204X, a model including both mutations was tested as previously described (18): yi = s0 + λgk + δhj + ui + εik, where γi is the record of individual i, s0 is the fixed effect of slaughter year d, λ is an indicator variable, which is 1, 0, or −1 depending on the genotype of individual i at locus NCAPG I442M, δ is an indicator variable, which is 1, 0, or −1 depending on the genotype of individual i at locus NCAPG I442M or GDF8 Q204X, hj is the additive allelic effect of allele j at locus GDF8 Q204X, ui is the infinitesimal genetic effect of individual i, and εik is the residual. Two likelihood ratio tests (A, likelihood of model with locus 1 and locus 2 vs. likelihood of model with locus 1; B, likelihood of model with locus 1 and locus 2 vs. likelihood of model without loci) were calculated to test for statistical significance.

Multiple testing within phenotype categories (growth, body composition, and lipid deposition, metabolites) had to be accounted for to avoid false positive associations. For this purpose, a false discovery rate (48) within each phenotype category (q-value) was calculated to account for multiple testing within phenotype categories (growth, metabolites, body composition, and lipid deposition). A level of α < 0.05 was considered an experiment-wise significant association.

A linear regression model was applied for the confirmation study in the Japanese Wagyu population: y = Xb + e, where y is the vector of phenotypic value, X is the design matrix of fixed effects composed of the number of NCAPG 442M alleles (0, 1, or 2) and covariates [age (in days) at the beginning of the test, sire, year, and season (summer or winter)], b is the vector of fixed effects, and e is the residual error. The proportion of variance explained by the NCAPG I442M locus was calculated as the difference between the variance explained by the above model and the variance explained by the model including only the covariates. The calculations were performed applying R statistical packages (http://www.r-project.org/).

RESULTS

Confirmation of NCAPG I442M and GDF8 Q204X allele effects on postnatal growth. Due to the experimental design of the F2 resource population that was established by means of embryo transfer into foster mothers followed by separation of mothers and calves immediately after birth, exclusively direct genetic effects on the growth of the offspring were monitored in the association study for postnatal growth. For the NCAPG I442M locus, an experiment-wise significant association (q < 0.05) between NCAPG I442M alleles and average daily gain was observed in the intervals 121–182 days, 183–273 days, and 274–365 days (Fig. 1A, Supplemental Table S4). The difference in weight gain between alleles 442I and 442M in the most distinctive interval 183–273 days amounted to 98 g/day. The NCAPG I442M locus explained 20.2% of the variance for average daily gain in this interval. In the first and last 3-mo intervals of observation, the effects on average daily gain associated with the two alleles were almost identical.

A confirmation study regarding the time point of the strongest differential growth associated with the NCAPG I442M mutation in the historically and geographically distant Japanese Wagyu population showed an analogous result: The effects of the NCAPG I442M mutation on average daily weight gain were time-specific (Fig. 1B), and the strongest allele-associated effect on postnatal growth was observed during the period of highest daily gain (see Supplemental Table S1).

The association analysis between the GDF8 Q204X mutation and postnatal growth resulted in an association pattern very similar to the one revealed for the NCAPG I442M locus in our experimental population: The interval 183–273 days displayed the strongest differences in average daily weight gain associated with the alternative GDF8 Q204X alleles (q < 0.006) (Fig. 1A, Supplemental Table S4).

Distinct effects of the NCAPG I442M and GDF8 Q204X alleles on plasma metabolites during puberty pinpoint divergent affected physiological pathways. A comprehensive targeted metabolome analysis focused on plasma lipid metabolites at the 240th day of life was undertaken to elucidate the relevant physiological processes involved in genetically determined divergent growth during the onset of puberty, the key interval for differential weight gain. A total of 229 plasma metabolites were identified and quantified, including 18 amino acids, free carnitine, 47 acyl-, hydroxyacyl-, and dicarboxylicarnitines, 79 glycerophosphatidylycholines, three prostanoids, arachidonic acid, docosahexaenoic acid, eight biogenic amines, 52 mono- and oligosaccharides, and 19 sphingomyelins (Supplemental Table S3). From the total of 229 metabolites, 28 had >5% missing data points, leaving 201 metabolites that were tested for their potential association with the NCAPG I442M and GDF8 Q204X alleles.

For the NCAPG I442M mutation, a significant (q = 0.013), positive effect of the 442M allele associated with arginine plasma level was observed (Fig. 2, Table 1). In contrast, there were no significant NCAPG I442M effects for the arginine precursors, its metabolites [citrulline, ornithine, or putrescine (Pnominal > 0.501), or for other amino acids in plasma (Supplemental Table S5)]. Dimethylarginines represent protein degradation products resulting from methylated arginine during intracellular protein turnover. Interestingly, our study revealed a suggestive (Pnominal = 0.004) effect of the NCAPG I442M locus on total dimethylarginine (TDMA); however, no significant effect on asymmetric dimethylarginine (ADMA) was observed. Consecutively, a refined association analysis with the difference between TDMA and ADMA concentration, which is supposed to reflect the symmetric dimethylarginine (SDMA) level, showed a highly significant result (q = 0.019). The allele NCAPG 442M, which is associated with a superior daily weight gain between 182 and 273 days of age and with elevated arginine levels, was also associated with an increased plasma SDMA concentration (Fig. 2). For the acylcarnitines, the NCAPG 442M allele showed a significant, positive association with plasma linoleylcarnitine (C18:2) level (q = 0.013). Ten additional nominally significant effects on acylcarnitines were obtained for the NCAPG I442M locus (Supplemental Table S5), predominantly for the C5–C8 and C16 fatty acid acylcarnitines.
For the $GDF8\ Q204X$ locus, a significant association ($q = 0.007$) with free carnitine level was observed (Table 1, Fig. 3): The $GDF8\ 204X$ allele, which is associated with increased body weight, was also associated with a decrease in the plasma level of free carnitine. In contrast to the $NCAPG\ I442M$ locus, where the glycerophosphatidylcholines and sphingomyelins seemed to be unaffected by the mutation, nominally significant effects on 22 glycerophosphatidylcholines and eight sphingomyelins were detected for the $GDF8\ Q204X$ locus (Fig. 4, Supplemental Table S5) with a concordant decreasing effect of the $GDF8\ 204Q$ allele for all respective metabolites. A significant effect was almost exclusively obtained for diacylglycerophosphatidylcholines, whereas lysoglycerophosphatidylcholines were mostly unaffected. In contrast to the $NCAPG\ I442M$ locus, there were no effects of the $GDF8\ Q204X$ locus on arginine, TDMA, or SDMA.

NCAPG I442M and GDF8 Q204X alleles are associated with particular effects on postnatal body composition and lipid deposition. Both loci, NCAPG I442M and GDF8 Q204X, showed a similar pattern with regard to allelic effects on quantitative postnatal body-mass gain (Fig. 1, Supplemental Table S4), while they exhibited substantial differences regarding effects on the plasma metabolites (Table 1, Supplemental Table S5). Therefore, a refined association analysis regarding effects on detailed body composition and lipid deposition at the end of the test period at 18 mo of age evaluated whether differences between loci existed not only with respect to the quantity but also to the quality of body-mass accretion.

The absolute and/or relative protein accretion in the carcasses ($CProt$, $CProtrel$) showed a highly significant, positive effect for those alleles associated with increases in body weight ($442M$ and $204X$) at the $GDF8\ Q204X$ locus ($q = 1.17 \times 10^{-8}$ ($CProt$) or $8.25 \times 10^{-7}$ ($CProtrel$, Supplemental Table S6)) and (less pronounced) at the $NCAPG\ I442M$ locus ($q = 6.14 \times 10^{-3}$, $CProt$). In contrast, the respective $NCAPG\ 442M$ and $GDF8\ 204X$ allelic effects on lipid deposition in the body had an opposite direction: The alleles at both loci were associated with a highly significant decreased fat content in the carcass [CFATrel: $q = 4.36 \times 10^{-3}$ ($NCAPG\ 442M$), $q = 2.84 \times 10^{-5}$ ($GDF8\ Q204X$)]. This resulted from a concordant decrease (Supplemental Table S6) in fat deposition in all lipid storage compartments [e.g., the percentage of subcutaneous fat in the carcasses (SFATrel, Fig. 5A)].

Whereas the NCAPG I442M and the GDF8 Q204X locus showed analogous effects on lipid and protein deposition in the carcass, further analyses of whole body composition revealed substantial differences between these loci. For example, the proportion of carcass weight relative to the empty body weight (CR) showed a highly significant association only with the GDF8 Q204X alleles ($q < 10^{-16}$), whereas no effect was observed for the NCAPG I442M locus (Fig. 5B). Furthermore,
the percentage of liver or kidney weight in carcasses (LIrel2, KIrel2) was not affected by the NCAPG I442M locus, whereas the effects of the GDF8 Q204X alleles were highly significant (Supplemental Table S6). These data indicate that GDF8 Q204X alleles specifically affect the growth of the carcass comprising mostly muscle, fat, and bone tissue, whereas for NCAPG I442M alleles, a proportional effect on entire body weight and carcass weight is exerted. In the two-locus model fitting NCAPG I442M and GDF8 Q204X effects, essentially identical effects regarding direction and magnitude of effects were obtained for both loci compared with the single locus models (Fig. 5).

Fig. 2. Effects of the NCAPG I442M alleles on arginine metabolism. Allele effects (± SE) of the NCAPG 442I allele are indicated in white columns, allele effects (± SE) of the 442M allele are indicated in black columns. Solid arrows designate metabolic substrate pathways; dashed lines designate a regulatory function. y-Axis displays plasma concentration of the respective metabolite in μM. All metabolites in shaded boxes (white letters indicating a significant association) were analyzed. P values indicate nominal significance for the difference in allele effect. ADMA, asymmetric dimethylarginine; SDMA, symmetric dimethylarginine; NOS, nitrogen oxide synthase; PRMT I, protein arginine methyltransferase type I; PRMT II, protein arginine methyltransferase type II; DDHA, dimethylarginine dimethylaminohydrolase.

Table 1. Experiment-wise significant associations of NCAPG I442M and GDF8 Q204X alleles with targeted plasma metabolites

<table>
<thead>
<tr>
<th>Trait</th>
<th>Mutation</th>
<th>LRT</th>
<th>( P_{\text{nomin}} )</th>
<th>( q )</th>
<th>( a_{\mu M} )</th>
<th>SE( a_{\mu M} )</th>
<th>% of Variance Explained</th>
</tr>
</thead>
<tbody>
<tr>
<td>C18:2</td>
<td>NCAPG 442M</td>
<td>14.90</td>
<td>( 1.33 \times 10^{-04} )</td>
<td>( 1.27 \times 10^{-02} )</td>
<td>0.000526</td>
<td>0.000151</td>
<td>16.5</td>
</tr>
<tr>
<td>Arginine</td>
<td>NCAPG 442M</td>
<td>14.12</td>
<td>( 1.72 \times 10^{-04} )</td>
<td>( 1.29 \times 10^{-02} )</td>
<td>9.04</td>
<td>2.34</td>
<td>8.2</td>
</tr>
<tr>
<td>SDMA</td>
<td>NCAPG 442M</td>
<td>12.80</td>
<td>( 3.46 \times 10^{-04} )</td>
<td>( 1.94 \times 10^{-02} )</td>
<td>0.1543</td>
<td>0.0433</td>
<td>11.2</td>
</tr>
<tr>
<td>Free carnitine</td>
<td>GDF8 Q204X</td>
<td>17.44</td>
<td>( 2.96 \times 10^{-05} )</td>
<td>( 6.65 \times 10^{-03} )</td>
<td>0.716</td>
<td>0.165</td>
<td>10.4</td>
</tr>
</tbody>
</table>

C18:2, linoleylcaritnine; SDMA, symmetric dimethylarginine; LRT, likelihood ratio test; \( P_{\text{nomin}} \), nominal P value; \( q \), false discovery rate accounting for multiple testing as determined by Storey and Tibshirani (48). \( a \) is the allele substitution effect allele 1 – allele 2. NCAPG I442M: allele 1 = 442M, allele 2 = 442I; GDF8 Q204X: allele 1 = 204Q, allele 2 = 204X. SE\( a \), standard error for \( a \).
Comparing the results from the association analyses for NCAPG I442M and GDF8 Q204X reveals that there is a strong similarity of associated effects regarding the key interval of divergent growth and relative content of body protein and body fat. However, the main difference in effects associated with the NCAPG I442M and the GDF8 Q204X mutation is the proportional or disproportional character of divergent carcass growth.

**DISCUSSION**

*Identification of the age at puberty as key interval for genetically divergent postnatal growth.* Our study investigated the effect of both the NCAPG I442M and GDF8 Q204X alleles on body-mass gain, plasma metabolite levels, and lipid deposition. Both the NCAPG I442M and GDF8 Q204X loci pinpointed the interval 183 and 273 days of age as the key interval for divergent growth in our resource population. In those European cattle breeds, which were founders of our experimental population, this interval includes the onset of puberty (6), a period that plays a central role in mammalian growth (1, 9). The results obtained in the experimental Charolais × German Holstein resource population regarding the key interval of divergent growth correspond to the data obtained from the Wagyu population. Considering that Wagyas are known for a later onset of puberty compared with Holstein or Charolais (11) when kept under similar conditions, the ontogenetic stage of the most distinctive growth associated effects of the NCAPG I442M mutation corresponded in both populations.

Our observation that the NCAPG I442M mutation predominantly acts at the time of most rapid growth during postnatal ontogenesis fits to the observation of Seipold et al. (44). Using the zebrafish model, the authors revealed that mutations in the NCAP genes predominantly affected rapidly amplifying progenitor cells compared with postmitotic cells. In our animal model, we postulate that there is a high proportion of proliferating cells during puberty, which represent the potential targets for NCAPG protein action. Considering our previous
results on birth weight (18), two other recent, conclusive reports of NCAPG locus effects on bovine growth (45, 46), and the results from the current study, there is a strong indication that indeed NCAPG, and specifically the I442M mutation, might be causal for genetic variation of pre- and postnatal growth, especially during onset of puberty. The confirmation study presented here is particularly relevant, because the concordant results were obtained in populations (Japanese Wagyu, European Holstein, European Charolais) that differ strongly in historical and geographical origin (34). Whether the NCAPG effects on postnatal growth are the consequence of prenatal NCAPG impacts, e.g., on fetal placentation (18, 19), or are due to genuine, independent postnatal effects will be of substantial further interest because in human, the effects of divergent prenatal development on postnatal phenotypes, especially on human lipid metabolism and obesity, are well established (49).

Increased endogenous plasma arginine level associated with the NCAPG 442M allele promoting proportional growth. The main distinctions between the effects associated with the NCAPG I442M and the GDF8 Q204X mutations are the proportional or disproportional character of divergent body growth. While the NCAPG I442M alleles exert a proportional effect on all body compartments, GDF8 Q204X alleles specifically affect the growth of the carcass by predominantly altering muscle, fat, and bone tissue. Results similar to the GDF8 Q204X mutation had been described in phenotypic studies on effects of the double muscling condition in the Belgian Blue cattle breed (5).

The differences between the effects of the GDF8 Q204X and NCAPG I442M mutations on individual compartmental growth are already reflected by a strong diversity in metabolic pattern associated with each mutation at 8 mo of age. Despite the effects of the NCAPG I442M mutation on plasma arginine concentrations, there was no difference between alleles regarding levels of ornithine (Fig. 2), a degradation product of arginine within the urea cycle. This indicates that inhibition of arginase activity might be the underlying mechanism for the divergent plasma arginine concentrations. Reports in the literature describe that a decreased arginase activity is a way to maximize the arginine supply in conditions of strong demand (59). The conditionally indispensable amino acid arginine is assumed to play a major role in growth, particularly during prenatal development (58). Arginine is described as an activator of the mTOR cell signaling pathway in skeletal muscle resulting in enhanced protein synthesis and whole body growth (61). Furthermore, arginine is the precursor of nitrogen oxide (NO), which has multiple important functions, e.g., in energy

![Fig. 5. Association of the NCAPG I442M and GDF8 Q204X mutation with body composition and lipid deposition. A: percentage of subcutaneous fat in carcass weight (SFATrel). B: percentage of carcass weight in empty body weight (carcass ratio, CR). The y-axis denotes the additive genetic allele substitution effect (± SE) of NCAPG 442M allele vs. 442I (indicated in black column) or the additive genetic allele substitution effect (± SE) of GDF8 204X vs. 204Q (indicated in gray column). The joint analysis: the model fits the NCAPG I442M + GDF8 Q204X effect vs. no locus effect. \( P_{\text{nominal}} \), nominal significance of the model; % variance explained, variance explained by the single locus or 2-locus model, respectively.](http://physiolgenomics.physiology.org/)

![Fig. 4. Effects of the GDF8 204Q allele on plasma glycerophosphatidylcholines and sphingomyelins. Effects of all glycerophosphatidylcholines and sphingomyelins showing a nominal significant \( P < 0.05 \) association are indicated. a204Q, Additive effect of the GDF8 204Q allele in relative units of phenotypic SD.](http://physiolgenomics.physiology.org/)

---

**Fig. 5. Association of the NCAPG I442M and GDF8 Q204X mutation with body composition and lipid deposition. A: percentage of subcutaneous fat in carcass weight (SFATrel). B: percentage of carcass weight in empty body weight (carcass ratio, CR). The y-axis denotes the additive genetic allele substitution effect (± SE) of NCAPG 442M allele vs. 442I (indicated in black column) or the additive genetic allele substitution effect (± SE) of GDF8 204X vs. 204Q (indicated in gray column). The joint analysis: the model fits the NCAPG I442M + GDF8 Q204X effect vs. no locus effect. \( P_{\text{nominal}} \), nominal significance of the model; % variance explained, variance explained by the single locus or 2-locus model, respectively.**

---

**Fig. 4. Effects of the GDF8 204Q allele on plasma glycerophosphatidylcholines and sphingomyelins. Effects of all glycerophosphatidylcholines and sphingomyelins showing a nominal significant \( P < 0.05 \) association are indicated. a204Q, Additive effect of the GDF8 204Q allele in relative units of phenotypic SD.**
metabolism by mediating insulin sensitivity and glucose uptake in skeletal muscle (31, 42). A decrease in arginase activity, as suggested by our metabolomic data, favors NO synthesis due to reduced competition of NO synthase (NOS) for arginine, and the elevated arginine levels themselves enhance NO synthesis by increasing inducible NO synthase mRNA translation (30, 60). Metabolomic data from mice highlight that wild-type mice have increased plasma arginine levels compared with type II diabetes mice (3), which is in agreement with an increased plasma arginase activity in human type II diabetes (25). Previous studies on the founder breeds of our animal model, Charolais and German Holstein bulls kept under environmental conditions identical to this study, demonstrated that the German Holsteins, which exhibited a decreased growth rate, an increased fat deposition, and a high frequency of the NCAPG I442M allele, also showed a significantly increased plasma insulin level at 8 mo of age (8, 18).

Whether arginine is the cause or effect of increased growth has to be evaluated carefully in our model. However, the elevated plasma arginine level observed in individuals carrying the NCAPG I442M allele is presumably not an unspecific result of an increased body-mass gain, because the GDF8 204X allele, also associated with a significant increase of postnatal growth in the respective time period, did not show any effects on plasma arginine levels. Furthermore, data on ADMA and SDMA indicate that specific mechanisms are in effect for the NCAPG I442M locus prohibiting increased levels of ADMA, because elevated ADMA concentration would exert a growth-impairing effect due to reduced NO synthesis from its arginine precursor. We can also conclude that the elevated arginine levels are not due to random protein degradation but specifically reflect a direct effect of the NCAPG I442M mutation on arginine metabolism itself, because there is no difference in the concentration of other amino acids in plasma of individuals with divergent NCAPG I442M genotype.

In addition to a direct effect on plasma arginine level, we identified a significant positive effect of the NCAPG I442M variant on TDMA, but no effect for ADMA (Fig. 2). Thus, the NCAPG I442M effect is obviously due to increased levels of SDMA, an isomer of ADMA. Because there is no synthesis of ADMA or SDMA from free arginine, the production of dimethylarginines is solely dependent on arginine methylation in proteins and proteolysis (33). Arginine is asymmetrically methylated by protein arginine methyltransferases type 1 (PRMT 1), whereas PRMT type 2 methylate arginine symmetrically (10). Protein arginine methylation is important for transcriptional regulation, RNA processing, and signal transduction (7). Data on ADMA and SDMA indicate that specific mechanisms are initiated by the NCAPG I442M locus that prohibit increased levels of ADMA, which might exert negative effects on growth due to a reduced NO synthesis. While ADMA is enzymatically degraded into citrulline and dimethylamines via dimethylarginine-dimethylaminohydrolases (33), SDMA is eliminated almost exclusively via renal excretion (26). Whether the difference in plasma levels of SDMA in our NCAPG I442M animal model is the result of divergent synthesis, liver reabsorption, or renal excretion remains to be elucidated.

In contrast to ADMA, a well-described, potent inhibitor of the NOS (39), which synthesizes NO from arginine, no analogous effect is described for SDMA. Thus, our data suggest that the increased arginine levels during a period of intensive growth are not results of NOS inhibition by elevated dimethylarginines. Only recent reports provided any indication of alternative functions of SDMA. These studies highlight the relevance of SDMA on the pathophysiology of stroke and cardiovascular disease (43, 53).

Free plasma carnitine levels associated with disruptive mutation in the myostatin-encoding GDF8 gene. In our resource population, free plasma carnitine at 240 days of age is significantly decreased in individuals carrying the GDF8 204X allele, which increases body weight and decreases body fat deposition. Free carnitine plays a major role during fatty acid transportation into the mitochondrion for β-oxidation and is involved in additional fatty acid, glucose, and nitrogen metabolic pathways. The impact of external dietary carnitine supplementation on mammalian growth and muscle development is controversially discussed (32). The agonistic signals from plasma fatty acids that lead to increased carnitine synthesis (40) might provide a link between plasma carnitine levels and the GDF8 Q204X mutation, because GDF8 knockout mice exhibit decreased free fatty acid concentrations (52). Although physiological effects of GDF8 mutations on muscle development and multiple metabolic pathways have been comprehensively reported (e.g., Ref. 12), no direct effect of myostatin on carnitine metabolism has been described. Thus, the question remains to be resolved, whether the variation in free carnitine level associated with the GDF Q204X mutation is a cause or consequence (via a yet unknown physiological mechanism) of alterations in myofiber or adipocyte development. However, the lack of association between free plasma carnitine and the NCAPG I442M allele indicates that the effect on free plasma carnitine is indeed locus specific and not related to generally divergent growth or lipid deposition. Because all individuals received a diet with an identical composition, a dietary effect on carnitine level seems also unlikely.

The decreased plasma concentration of glycerophosphati-dylcholines and the increased lipid deposition associated with the GDF8 204Q allele are analogous to reports about similar effects observed in mice treated with Rosiglitazon, which fosters lipid deposition in adipose tissues (3). Alternatively, the concerted increase in glycerophosphatidylcholines that was associated with the GDF8 204X allele might also be due to an elevated level of phospholipids in muscle tissues, which has been described in individuals with a mutation in the GDF8 gene (14). Glycerophosphatidylcholines are the most common phospholipids in mammals. In higher organisms, perturbation of glycerophosphatidylcholine synthesis can lead to inhibition of growth or even cell death (13). Thus, the increased glycerophosphatidylcholine levels in plasma associated with the GDF8 204X allele might also specifically reflect the increased muscle growth associated with this allele.

Metabolic biosignatures mirror particular growth pattern. In conclusion, our study, which to our knowledge is the first combining targeted comprehensive metabolomic plasma analysis and genetic association studies in a large animal species, discriminates distinct metabolomic biosignatures associated with two genetic loci exhibiting major effects on pre- and/or postnatal growth. For NCAPG, a previously unknown, conclusive link could be drawn to the arginine metabolism, which is crucial for many regulatory mechanisms in growth and energy metabolism. Furthermore, our study of the GDF8 Q204X
mutation indicated novel pathways that seem to be affected by a functional deficit in the growth-inhibiting factor myostatin. The hypothesis of different physiological processes involved in the action of the two loci is supported by the observation that the NCAPG I442M and GDF8 Q204X mutations showed independent effects on growth as demonstrated by the results from the single- and two-locus association models. The divergent, locus-specific metabolomic patterns at the onset of puberty were mirrored by particular proportional or nonproportional modulations of compartmental growth and lipid deposition associated with the two mutations. Interestingly, our genetic analyses highlighted associations between the NCAPG I442M or GDF8 Q204X locus, respectively, and two metabolites (carnitine, arginine) that are frequently discussed as feed additives promoting growth and physical performance in, e.g., cattle and humans (51, 58). Our resource population provides a valuable, uniparous large animal model to survey these specific effects of both genes, GDF8 and NCAPG, regarding the underlying functional mechanisms during pre- and postnatal growth and lipid deposition.

ACKNOWLEDGMENTS

We thank our colleagues at the FBN Dummerstorf who helped in the generation and care of the SEGFAFM 2 resource population for their continuous support of our work. The technical support from A. Kühnl, S. Wölhl, P. Widmann, A. Eberlein, A. Schulz, C. Fiedler, and I. Rothe is also thankfully acknowledged. Dr. R. Pühl provided valuable information on carcass dissection traits.

GRANTS

The work was supported by the German Federal Ministry of Education and Research (BMBF) within the scope of the FUGATO QUALIPID project (FKZ 0313391C).

DISCLOSURES

This study was conducted under the direction of the Leibniz Institute for Farm Animal Biology (FBN), Dummerstorf/Germany, independently of Biocrates, which provided the metabolomics measurements on a fee-for-service basis. FBN researchers did not receive any financial advantages (e.g., consultancy fees) from this company. There are no further competing interests to declare.

REFERENCES


