Single Nucleotide Polymorphisms in *CLDN14* and *SMOC1* Affecting Bone Mineral Density Influence Other Musculoskeletal Traits

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Introduction

Bone mineral density (BMD) is the result of a complex interaction between genes and environmental factors. As a metric, it can be used to identify osteoporosis, determine the risk of fracture, and measure responses to treatments that affect bone remodeling. Previous research has shown BMD to be highly heritable with genetic factors accounting for approximately 50% to 85% of BMD (Özbaş, 2012). Other anthropometric traits have similar genetic components: heritability of lean body mass, for example, has been shown to be up to 60-90% (Karasik & Kiel 2008). It is well established that muscle, bone, and fat composition are all closely associated. Higher lean body mass has been related to BMD and decreased fracture risk in elderly men (Verchueren et al., 2013) and recent studies have shown that increased lean mass and decreased fat mass raises BMD and reduces fracture risk in post-menopausal women (Sotunde et al., 2015). As novel single nucleotide polymorphisms (SNPs) influential for bone development continue to be identified, it is important to determine whether these SNPs have the same effect on musculoskeletal traits.

Zhang et al. (2014) performed a genome-wide association study (GWAS) to identify which genes influenced BMD. This three stage GWAS confirmed the genome-wide significance (GWS) of 13 previously reported loci and identified two novel loci at the GWS level: rs227425 in the SPARC Related Modular Calcium Binding 1 gene (*SMOC1*) was significantly associated with BMD, and rs170183 in the claudin 14 (*CLDN14*) gene was also significantly associated with BMD, though only among females.

The SMOC1 protein has been identified as an important contributor to bone development and quality. Choi et al. (2010) discovered that loss of SMOC1 results in inhibited mineralization and decreased expression of osteoblast differentiation markers, while Okada et al. (2011) determined *SMOC1* expression to be essential for skeletal formation, especially for limb development. *CLDN14* regulates Ca^{2+} transport and paracellular permeability at epithelial tight junctions. Knocking out *CLDN14* leads to hypermagnesemia, hypomagnesiuria, and hypocalciuria under increased Ca^{2+} intake conditions, implicating a role for this gene in musculoskeletal functioning.

Prior studies have demonstrated that genetic variants located in the *SMOC1* and *CLDN14* genes affect bone and limb development. However, research has yet to incorporate these genes or their gene products into the discussion of muscle-bone crosstalk and body composition. Therein, the purpose of this study was to determine whether these SNPs (rs170183 in the *CLDN14* gene and rs227425 in the *SMOC1* gene) are associated with various musculoskeletal traits in a cohort of African American children and a Caucasian young adult cohort undergoing resistance training in the non-dominant arm.

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Experimental Design and Methods

Cohorts:

FAMuSS Cohort

The Functional Single Nucleotide Polymorphism Associated with Human Muscle Size and Strength (FAMuSS) study examined a cohort of Caucasian young adults (mean age=24.5) who volunteered for a 12-week unilateral resistance training intervention of the non-dominant arm. At entry and exit several traits were measured including 1-RM strength, maximum voluntary contraction (MVC) and whole muscle volume (pre-training, post-training, and % change). At entry and exit MRIs of both the dominant and non-dominant arm were analyzed for tissue volumetric measures using modified RAPIDIA® software.

Bone Health Cohort

The Bone Health Cohort consists of 150 African American male and female participants who were enrolled at Children's National Health System as part of a fracture analysis study examining dietary intake of calcium and vitamin D and collecting various measurements of bone morphometry and obesity. The cohort consists of 75 participants with a forearm fracture and 75 controls without any history of fracture ranging from five to nine years of age. Measurements of participants' total body-less head bone mineral density (tBMD), lumbar BMD (lBMD), total body-less head BMC (tBMC), and lumbar BMC (lBMC) were obtained using a hologic dual-energy x-ray absorptiometry (DXA) scan. Exclusion criteria for the study included underlying bone mineralization disorders, use of antiepileptic medications, current or past use of daily oral steroids for >1 month, or a chronic illness that interacted with bone density such as sickle cell disease, cancer, kidney disease, malabsorptive disease, or cerebral palsy.

Genotyping:

Genomic DNA was extracted from whole blood samples using the PUREGENE DNA Purification System (Gentra Systems, Minneapolis, MN) per manufacturer instructions. Genotypes were determined with TaqMan allelic discrimination assays via the 5' nuclease activity of Taq polymerase to detect a fluorescent reporter signal released during a PCR reaction. The final working solution for each SNP contained 3 µL of DNA (2 ng/µL) and 5 µL of combined primers, probe, and Taqman® Universal PCR Master Mix (Life Technologies, Carlsbad, CA). PCR was performed on thermal cyclers using a profile of 10 minutes at 95˚ C for denaturation, 44 cycles of 15 seconds at 95˚ C, and 1 minute at an annealing temperature of 60˚ C, followed by a brief cooling period. The post-PCR allele calling was completed using a Real Time PCR 7900HT (genotyping software SDS version 2.4) and confirmed manually. Genotypes were determined by comparing the ratio of fluorescence signals (fluorophores: VIC/FAM).

Table 1

RS Number	Gene	Location	Allele Frequency by Cohort		Allele frequency (1000genomes)		HWE p-value
			Bone Health	FAMuSS	All	African-American	Bone Health/ FAMuSS
Rs227425	*SMOC1*	Chr.14: 70456699	T/G (.53/.47)	T/G (.51/.49)	T/G (.53/.47)	G/T (.65/.35)	0.22/0.90
Rs170183	*CLDN14*	Chr.21: 37848334	G/A (.86/.14)	A/G (.52/.48)	A/G (.36/.64)	G/A (.95/.05)	0.06/0.09

Statistics:

A χ^2-test was used to compare observed genotype frequencies to those expected under Hardy-Weinberg equilibrium (Table 1). All phenotypes were assessed for normality using the Shapiro-Wilk test. In the Bone Health Cohort, genotypes were tested for associations with lean mass, fat mass, % fat (all calculated by removing the head, as this skews results in pediatric populations), and physical activity measured by total hours of sun exposure per week. The A allele of the *CLDN14* SNP rs170183 is very rare in African Americans. Therefore, as the Bone Health Cohort consisted of pediatric African Americans, we did not have any AA individuals with which to test associations. Phenotypes assessed in the Bone Health cohort were analyzed with an ANCOVA (age and sex covariates), followed by *post-hoc* pair-wise comparisons to identify significance between individual groups. SNPs were assessed for associations between genotype and 1- repetition max (1-RM) strength, maximum voluntary contraction (MVC) and whole muscle volume (pre-training, post-training, and % change) in the FAMuSS cohort. The FAMuSS cohort was initially stratified by sex and then significant associations were assessed using analysis of co-variance (ANCOVA) models with age and weight as covariates. All group analyses that were significant were followed by post-hoc t-tests to determine individual group significance. Data are reported as means ± SEM or adjusted means ± SEM where appropriate. Cutoff for significance was $P < 0.05$.

Results

In the FAMuSS cohort, rs170183 was significantly associated with pre-exercise MVC strength (p=0.0100), post-exercise MVC strength (p=0.0164), and % change in whole muscle volume (p=0.0413) in females. This SNP was also nearly significant in the % change in 1-RM strength (p=0.0800) in female participants only (Table 2).

In the Bone Health cohort, rs170183 (p=0.0600) and rs227425 (p=0.0419) were associated with total body fat mass across both sexes (Table 3).

Table 2: Significant Associations in the Analysis of CLDN14 (rs170183) and SMOC1 (rs227425) in the FAMuSS cohort

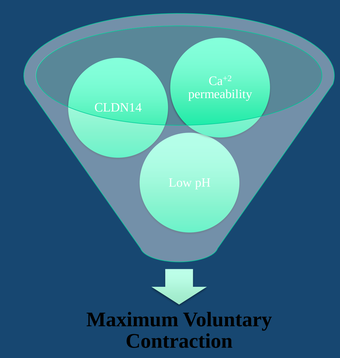
SNP	Phenotype	Group	P-value	Genotype	N	Adjusted mean ± SEM	P-values for individual group comparisons
CLDN14 (rs170183)	Pre-exercise MVC strength (lbs.)	Females	0.0100	AA	106	69.4 ± 2.4	*p=0.0238
				AG	201	62.7 ± 1.7*	
				GG	94	70.8 ± 2.5*	
CLDN14 (rs170183)	Post-exercise MVC strength (lbs.)	Females	0.0164	AA	106	85.4 ± 2.7*	*p=0.0265
				AG	201	76.6 ± 2.0*	
				GG	94	83.5 ± 2.9	
CLDN14 (rs170183)	Change in 1-RM strength (%)	Females	0.0800	AA	107	67.2 ± 3.5	NONE
				AG	198	67.0 ± 2.5	
				GG	97	57.8 ± 3.5	
CLDN14 (rs170183)	Change in whole muscle volume (%)	Females	0.0413	AA	73	11.6 ± 1.0	NONE
				AG	139	10.9 ± 0.7	
				GG	67	8.3 ± 1.0	

Table 3: Significant Associations in the Analysis of CLDN14 (rs170183) and SMOC1 (rs227425) in the Bone Health cohort

SNP	Phenotype	P-value	Genotype	N	Adjusted mean ± SEM	P-values for individual group comparisons
CLDN14 (rs170183)	Total body fat mass (g)	0.0600	AA	0		NONE
			GG	74	7699 ± 622	
			GA	27	9370 ± 865	
SMOC1 (rs227425)	Total body fat mass (g)	0.0419	GG	29	7735 ± 802	*p=0.0475
			GT	51	9133 ± 714*	
			TT	20	6589 ± 991*	

Discussion

- ***Across both sexes, rs170183 of the CLDN14 gene and rs227425 of the SMOC1 gene were both associated with total body fat mass in the Bone Health cohort.***
- Significant associations were discovered between muscle strength and rs170183 among females only in the FAMuSS cohort. rs170183 also trended toward a significant association between genotype and change in strength (% change in 1-RM strength) once more in female participants.
- While prior research shows no direct association between *SMOC1* and total body fat mass, *SMOCI* is a SPARC-related gene. ***Previous research suggests that VEGF, a signaling protein affected by SPARC, does in fact affect body fat. This link makes further investigation of SMOC1 as it relates to the development of body fat a promising future research direction***.
- A 2014 GWAS identified that a SNP in the *CLDN14* gene, rs170183, was associated with total hip, lumbar spine, and femoral neck BMD in females (Zhang et al. 2014). In Zhang et al.'s (2014) study, the G allele was associated with increased bone mineral density across all sites. Other studies have found that the *CLDN14* gene is associated with renal calcium handling and serum parathyroid hormone (Thorleifsson, Gong).
- ***Our results support the sexually dimorphic effect of the rs170183 SNP as significant associations were only found in the female population of the FAMuSS cohort.***
- Thorleifsson et al. (2009) have also discovered an association between the *CLDN14* gene and serum total CO_2 whereby individuals carrying one copy or more of the C allele of rs219780 experienced decreases in total serum CO_2 levels.
- Increased levels of CO_2 lead to a decrease in blood pH (acidosis), with the resulting blood pH affecting muscle performance. Skeletal muscle contraction occurs due to an increase in cytoplasmic Ca^{2+} concentrations, which causes Ca^{2+} to bind to troponin C, leading to the actin-myosin interaction responsible for muscle performance (MacIntosh 2003). Therein, pH has been shown to modulate Ca^{2+} sensitivity: maximum force and Ca^{2+} permeability both decrease with a lowering of pH and acidosis is a major contributor to muscle fatigue (MacIntosh 2003).
- As *CLDN14* is associated with increased levels of CO_2 which reduce blood pH, and maximum muscle force and Ca^{2+} permeability are attenuated by a lowered pH, the link between rs170183 of the *CLDN14* gene and maximum voluntary contraction (MVC) determined in this study becomes more relevant.



- ***The relationships among CLDN14, serum CO_2, and maximum muscle force established in prior research suggest that the association between rs170183 of the CLDN14 gene and maximum voluntary contraction observed in this study is supported by the literature and promotes research regarding the role of CLDN14 in muscle development***.
- Since the SNPs investigated have not yet been linked to functional outcomes, this remains an area for further study. Future studies may also want to further explore the association between rs17013 and MVC seen in this study to better understand the mechanism behind this link, especially in light of the *CLDN14* gene's effect on CO_2 levels. In addition, future studies may further investigate the sexual dimorphism displayed in the effects of rs170183 on musculoskeletal traits.
- ***To our knowledge, this is the only study to associate these two SNPs with musculoskeletal traits other than BMD.***

Figure 1: Image of a pediatric patient undergoing a DXA scan.
Source: http://pediatrics.med.miami.edu/crunchtime/facilities/dxa-laboratory

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