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RESEARCH ARTICLE

Untargeted Serum Proteomics Profiling in Female Patients with Idiopathic Scoliosis

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ABSTRACT

Background: Idiopathic scoliosis is a common structural spine curvature of unknown etiology. Scoliosis onset and progression are likely related to the interplay between genetics and the environment. Serum protein levels are influenced by both genetic and environmental factors and thus, are a promising methodology.

Aims: We aimed to determine whether serum protein levels differed between idiopathic scoliosis cases and controls. In scoliosis cases, we also aimed to determine if protein levels were correlated with curve severity.

Methods: In the discovery population, serum blood samples were obtained from 7 females with severe scoliosis and 11 unaffected female controls. Liquid chromatography-mass spectroscopy was used to quantify protein levels. Wilcoxon rank sum tests were used to test for differences between cases and controls. Within scoliosis cases, Spearman correlation coefficients were used to test the correlation between curve severity and age-adjusted protein levels. Candidate proteins were defined as proteins that significantly differed between cases and controls and were moderately or strongly correlated with curve severity (ρ>0.30). We validated candidate proteins using the SomaScan Discovery v4.1 proteomics platform with serum from 11 females with scoliosis.

Results: In the discovery analysis, 10 proteins differed between cases and controls ($p<0.05$) and were correlated with protein levels (ρ=0.36-0.65). Of these candidate proteins, one protein, alpha 2-HS glycoprotein (AHSG), was significantly correlated with curve severity ($p=0.0323$) in the validation population. Fixed effects meta-analysis showed a strong inverse correlation between curve severity and decreasing alpha 2-HS glycoprotein levels (meta-combined ρ-0.67, $p=0.0052$).

Conclusion: Alpha 2-HS glycoprotein met our criteria for significance in both the discovery and validation populations. Developing a proteomic signature for idiopathic scoliosis has important clinical implications. This study supports the feasibility of untargeted proteomics and provides evidence for the potential role of alpha 2-HS glycoprotein levels in scoliosis etiology.

INTRODUCTION:

Idiopathic scoliosis (IS) is a complex three-dimensional structural spinal curvature of ≥ 10 degrees with vertebral rotation. Approximately 1-3% of normal pre-pubescent and adolescent individuals[1,2] will have a spinal curvature, of which 0.3-0.5% of these individuals will have curvatures of >20 degrees, drawing potential recommendations for treatment. Progression to severe curves of >40 degrees is more common among adolescent females at a ratio of 7.2:1[3]. Conservative management for affected individuals is limited. Bracing has been confirmed to decrease or stop curve progression in some immature individuals with minor or moderate curves[4]. The effectiveness of therapeutic programs such as core stabilization exercises and the Schroth methodology to prevent curve progression is still debated within the literature[5]. Severe curves at skeletal maturity are associated with significant long-term health issues including back pain, cardiopulmonary issues, and cosmetic concerns [6-10]. As an adolescent with a high probability of curve progression, surgical intervention may be recommended as the only option. Although the surgery is well tolerated, it can lead to significant short and long-term health care costs as well as potential functional morbidities over the course of a lifetime[11,12]. As a result, there is a strong need to develop novel treatment interventions that address the underlying etiology of IS at an earlier age, ideally before severe curve progression.

Despite significant effort, the etiology of IS remains unknown. The familial nature of scoliosis as well as its genetic heritability have been well studied[13-16]. However, genetic association studies have been hampered by the complex nature this disorder which is exemplified by the substantial heterogeneity in IS disease presentation across affected individual[17]. Genetic variants in or near *LBX1* and *GPR126* have been consistently associated with IS in both whole exome sequencing (WES) and genome-wide association studies (GWAS)[18,19]. However, these variants only explain a modest proportion of variance in the disease and moreover, the functional role of these variants within IS disease pathways remains poorly understood[14,17]. As a result, it is hypothesized that curve onset and progression are related to both genetic and environmental factors[20-31]. Serum protein levels are influenced by genetics and environment, and thus are a promising avenue to study IS etiology. Plasma protein measurements are the most widely used diagnostic procedure in medicine. Molecular markers can reflect a defined phenotype, the progression of disease, clinical responsiveness to interventions, and can aid in providing patients with personalized treatments. To date, the identification of protein molecular signatures has given significant insight into the etiology of numerous complex disorders (e.g., Parkinson's disease [32], Alzheimer's disease [33]) and has aided in the discovery of potential drug targets (e.g. Type 1 Diabetes [34]).

To gain possible insight into the mechanisms that potentially correlate with scoliosis curve severity, we conducted a pilot study to evaluate the plasma proteomic levels in individuals with IS. We tested the serum protein levels among females with IS compared to controls who were frequency matched based on age and sex. Among the IS cases, we also tested whether differential protein expression was correlated with curve severity.

METHODS:

Discovery Population: Serum blood samples were obtained from n=7 females with IS (Cobb angle range 46-85°) and n=11 unaffected females in our discovery population (see Table 1). Proteomics profiling was performed using untargeted LC-Mass Spectroscopy (Orbitrap Fusion mass spectrometer with an Easy-nLC 1200 system, Thermo Fisher Scientific). Proteins had to be present above the detection threshold in a minimum of 50% of the cases or controls to be included. Missing values were imputed using the one-half minimum value method[35]. All samples were normalized using the stabilized variance transformation method[36]. Linear regression models were used to regress out age from the protein levels. Wilcoxon rank sum tests were then used to test for differences in age adjusted protein levels between cases and controls. Within cases, Spearman correlation coefficients were used to test the correlation between curve severity and age adjusted protein levels. Proteins were considered candidates if (1) protein levels were significantly different between cases and controls and (2) there was evidence of a moderate or higher correlation (|Spearman rho>0.30|) between curve severity and protein levels. Given the small sample size, we used a two-sample validation design to improve the rigor of this untargeted work by testing the correlation between these candidate proteins and curve severity in a separate, validation population.

Validation Population: Serum samples were collected from n=10 females with IS (Cobb angle range: 24-85°) in the validation population. Protein levels were measured using the SomaLogic SomaScan® Discovery assay, version 4.1 (SomaLogic, Inc.). SomaScan uses *SOMAmers*, high-affinity, highly specific aptamers that bind to specific protein structures to quantify protein levels [37]. Initial processing and protein quantification (done by SomaLogic) consisted of plate hybridization to control for variability across array signals, median signal normalization to control for technical variability of replicates within a run, and plate scaling and calibration of SOMAmers to control for inter-assay variation between analytes and batch differences between plates. Additionally, adaptive normalization by maximum likelihood were used to remove edge effects and technical variance. Protein levels were measured as relative frequency units (RFUs), which were log normalized for downstream analysis. Next, linear regression models were used to regress out the effect of age on protein levels. Spearman correlation coefficients were used to test the association between curve severity and age adjusted protein levels. Candidate proteins were confirmed if the direction of correlation between curve severity and protein levels was consistent in both the discovery and validation populations and the Spearman correlation coefficient was significantly correlated with curve severity in the validation population ($p<0.05$). We used a fixed effects meta-analysis to combine the correlation coefficients for all proteins identified as candidates in the discovery and confirmed in the validation populations. The spearman correlation coefficients were meta-analyzed using the 'metafor' R (v 4.2) package[38].

RESULTS:

In the discovery analysis, 187 proteins met quality control (QC) criteria. Of the 187 proteins that met the filtering criteria, 18

proteins were significantly different between cases and controls ($p<0.05$). Of the 18 proteins, 10 proteins were also moderately or strongly correlated (Spearman rho: 0.36 to 0.65) with curve severity (See Table 2). 8 of these 10 proteins were available for testing in the validation population (see Table 2). We identified a single protein, alpha 2-Heremans-Schmid glycoprotein (UniProt #P02765), also known as fetuin-A, that differed significantly between cases and controls ($p=0.0268$) and was strongly negatively correlated with curve severity in both the discovery and the validation populations (Table 1 and 2). Fixed effects meta-analysis was used to combine the correlation coefficients from the discovery and validation populations (combined Spearman correlation coefficient: -0.67, 95% CI: -0.24 to -0.88, $p=0.0052$), indicating a strong inverse correlation between increasing curve severity and decreasing AHSG levels (Figure 1). One additional protein, Coagulation factor VII (Uniprot ID# P08709), was significantly different between cases and controls and was correlated with curve severity in both the discovery and validation populations. However, the direction of correlation between curve severity and Coagulation factor VII levels was discordant in the discovery population (positively correlated with curve severity) compared to the validation (negatively correlated with curve severity) population and thus, did not meet our candidate criteria (see Table 2).

Table 1. Comparison of demographics and clinical characteristics in the study populations

	Discovery Population				*Validation Population*	
	Case (n=7)		Control (n=11)		Case (n=10)	
	n | mean	% | stdev	n | mean	% | stdev	n | mean	% | stdev
Female Sex, n (%)	7	100%	11	100%	8	100%
Age, mean (stdev)	14.1	2.1	13.9	2.1	9.65	1.06
Curve Severity, mean (stdev)	58.0	13.3	NA	NA	56.3	16.6

Table 2. Summary of protein candidates tested in the validation population

	Discovery Population			Validation Population	
Protein	Fold Change†	Wilcoxon P	Spearman Rho*	Spearman Rho*	Spearman P Value
Alpha 2-HS glycoprotein (AHSG)	1.19	**0.0268**	**-0.65**	**-0.67**	**0.0323**
Apolipoprotein B	1.35	0.0041	-0.36	-0.24	0.5096
Cholesteryl ester transfer protein	0.78	0.0441	-0.65	-0.40	0.2584
Complement factor H-related protein 1	1.30	0.0204	+0.36	+0.55	0.0972
Coagulation factor VII	0.78	0.0012	-0.60	+0.72	0.0195
Insulin-like growth factor-binding protein 4	1.23	0.0346	+0.42	+0.02	0.9468
Protein-glutamine gamma-glutamyltransferase E	0.85	0.0154	-0.38	+0.05	0.8939
Zymogen granule membrane protein 16	0.88	0.0268	-0.45	+0.46	0.1854
Fibrinogen Alpha Chain	1.16	0.0441	-0.38	NA	NA
Hornerin	1.48	0.0114	+0.44	NA	NA

*†Fold change for IS cases versus age matched controls, *Spearman Rho = correlation coefficient representing correlation between protein levels and curve severity, a positive (+) correlation coefficient indicates that protein level increased as curve severity increased, a negative coefficient (-) indicates that protein level decreased as curve severity increased. AHSG, emboldened, was the only protein candidate that was confirmed in the validation population, NA = protein not available for testing in validation sample.*

Figure 1. Inverse correlation between AHSG protein levels and IS curve severity in both the discovery and validation populations

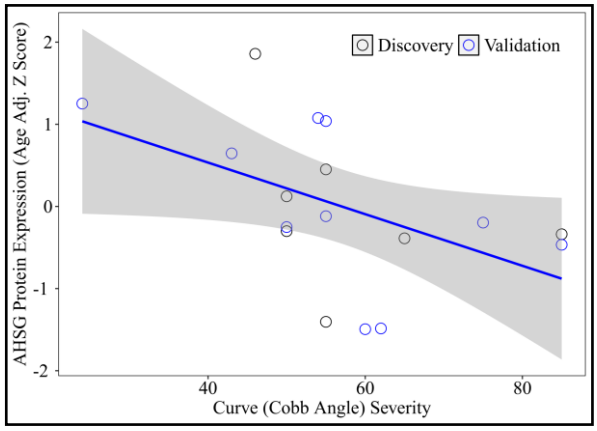

Figure 1 Description: Protein levels in the two populations (discovery = black dots and validation = blue dots) were standardized (z-scores, y-axis) and a fixed effects meta-analysis was used to combine slopes (blue line represents average slope, gray area represents 95% confidence interval). Increasing Cobb angle (x-axis) was correlated with lower protein levels (y-axis).

DISCUSSION:

We identified a single plasma protein candidate, alpha 2-HS glycoprotein, a protein product of the alpha 2-HS glycoprotein gene (*AHSG*, See Figure 2) that was associated with IS. Based on untargeted proteomic mass spectroscopy, this protein was significantly different between IS cases and sex matched controls and was also correlated with IS curve severity in the affected cohort. We validated this finding with the SomaScan assay in a separate sample of IS female patients, confirming an inverse relationship between AHSG protein expression and curve severity.

Figure 2. AHSG Gene Transcripts and Location within Chromosome 3

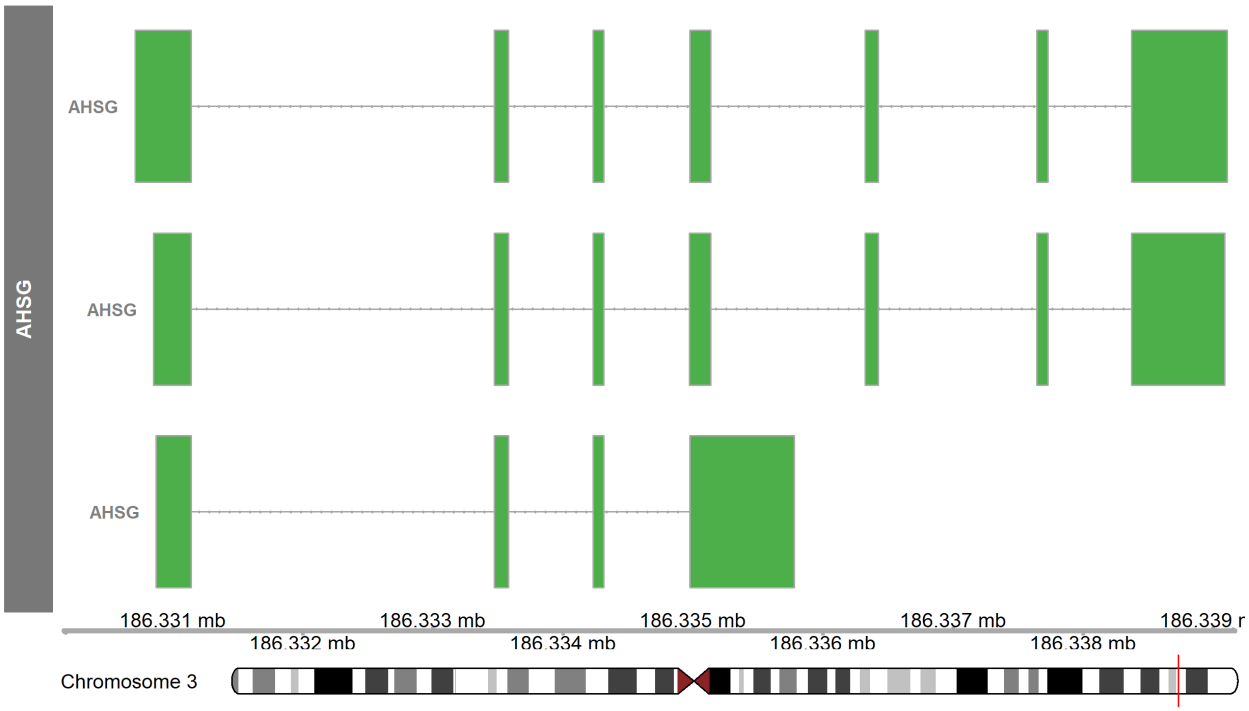

Figure 2 Description: AHSG gene transcript isoforms (top panel) and location of gene on chromosome 3 (vertical red line, bottom panel).

Alpha 2-HS glycoprotein, also known as Fetuin-A, is a negatively charged carrier glycoprotein belonging to the cystatin family of cysteine protease inhibitors with a domain of conserved cysteine residues which enables them to inhibit protease activity. The protein is primarily synthesized in embryonic cells and hepatocytes with additional synthesis in adipocytes and inflammatory cells[39,40] . As the primary candidate from this investigation, AHSG is transcribed as a single chain preprotein comprised of a heavy A-chain and a light B-chain with a connecting single disulfide peptide bridge between the two chains[40]. Posttranslational modification of AHSG is not clearly understood, but has been shown to occur with both chains, involving glycosylation, proteolytic cleavage, and phosphorylation to form an 'active' AHSG[40]. The secretion and release of AHSG has been shown to be affected by multiple exogenous factors[39,40]. As an active multifunctional protein within multiple physiological pathways, AHSG is present in nearly all vascularized tissues with well-established roles in regulation of calcium and bone metabolism, insulin signaling regulation, inflammation, vascular calcification, and adipogenesis[39-42]. A knockout AHSG mouse model revealed age dependent effects on skeletal development[41]. At birth, the skeletal system of AHSG (-/-) mice were phenotypically similar to the AHSG (+/+) control mice. However, as the mice aged, the knockout mice had distinct skeletal changes including delays in growth plate maturation, changes in femoral growth trajectory, and increased bone thickness. This mouse model established AHSG as a factor in post-natal musculoskeletal development. The potential relationship between AHSG and IS, as a phenotypic disorder of the musculoskeletal system, needs to be explored, as to whether this plasma protein may mirror the physiological state of the scoliotic condition and/or progression.

The top protein in the current study, AHSG, also has a role in lipid metabolism[39,43]. In the presence of AHSG the uptake and storage of free fatty acids increases in adipocytes in the liver suggesting a positive correlation between AHSG levels and fatty liver disorders and obesity[43]. Body mass has been shown to be altered in IS[6]. A recent mendelian randomization study confirmed a causal association between BMI and IS[44]. Increasing BMI was inversely associated with IS prevalence (inverse variance weighted estimate: beta = -0.56, $p=1.8x10^{-3}$). Furthermore, a case control analysis of serum metabolomic profiles revealed increased lipid metabolism in IS cases versus controls[45]. Collectively, our proteomics work in combination with prior studies linking IS to BMI and lipid metabolism suggest a potential interrelationship between lower AHSG levels, lower BMI, and increased IS severity; a hypothesis that should be rigorously tested in follow-up studies.

The current work is complemented by a select number of quantitative proteomic studies[46-48]. Makino et al[47] and Wang, et al[48] explored the plasma or serum proteome in varying IS study cohorts versus controls. Makino et al[47] found vitamin D binding protein (GC) significantly correlated with curve severity (Cobb angle) in individuals with thoracolumbar/lumbar curve. GC was not included in the 187 proteins that our filtering criteria and thus, was not evaluated in the current study. In wang et al[48], enrichment analysis of proteins that were

significantly different between IS cases and controls identified several significantly enriched pathways related to blood clotting/coagulation including coagulation cascades, platelet aggregation, platelet activation, and blood coagulation pathways. In our discovery analysis, coagulation factor VII, activator of coagulation pathways[49], was significantly different between cases and controls and was correlated with curve severity. Although coagulation factor VII did not meet our candidate criteria, this protein should be investigated in subsequent work. Collectively, prior studies including our current work are indicative of the potential of proteomics as a tool for studying the etiology of IS. However, the marked difference in results is indicative of the difficulties in the study of proteomics and the influence of confounding factors. Further longitudinal studies are needed to determine how plasma levels of candidate proteins identified in our work, specifically AHSG, as well as other related work influence IS curve onset and progression.

In the current study there are several potential limitations. First, the collection of the serum samples from the IS cases as compared to the controls were performed under varying conditions. The samples from the IS cohort were primarily collected during a fasting state (prior to surgery), whereas the controls were not (clinic visit). Differences in fasting between cases and controls may explain the interesting pattern in the results; AHSG levels were higher in cases versus controls but were negatively associated with curve severity. Based on the cross-sectional nature of data collection following curve progression, correlations with curve severity in the plasma could be causative or secondary to curve progression. Lastly, the representative cohorts were extremely small in number. Despite these potential limitations, the two-stage study design with secondary confirmation of a single protein expression in an independent IS cohort increases the rigor of our experimental design.

CONCLUSION:

Development of a reproducible proteomic signature predictive of IS onset and/or progression has important implications for the development of clinical screening tools as well as the identification of novel therapeutic strategies. In this preliminary work, AHSG met our criteria for significance in both the IS discovery and validation populations. This pleiotropic protein is an intriguing candidate based on its role in bone growth and remodeling via mineralization and lipid hormone signaling pathways[39]. Further work is needed to understand the potential mechanisms connecting plasma levels of AHSG to IS curve progression and more importantly, whether alterations in AHSG levels precede curve onset. Our results support proteomics as a valuable methodology for studying IS etiology and curve progression.

CONFLICTS OF INTEREST:

In the previous 24 months, PC was a paid consultant for American Orthopaedic Foot and Ankle Society.

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