TRANSLATIONAL IMMUNOMETABOLISM RESEARCH IN ADOLESCENT IDIOPATHIC SCOLIOSIS

THE FEASIBILITY OF MECHANISTIC STUDIES ON THE ROLE OF IMMUNITY IN ADOLESCENT IDIOPATHIC SCOLIOSIS

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Descriptive Note

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TITLE: Establishing the Feasibility of Mechanistic Studies on the Role of the Immune System in Adolescent Idiopathic Scoliosis

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Abstract

Background: The most prominent form of spinal curvature in youth (scoliosis) is Adolescent Idiopathic Scoliosis (AIS). While AIS aetiology is unclear, the role of paraspinal muscle in its genesis has been debated, as These muscles provide spinal stability and motion. It is known that paraspinal muscle (PM) exhibits differential fibrosis on both sides of the spine. As fibrosis is the result of immune system activation, we sought to elucidate the upstream mechanisms of immune-muscle interactions in AIS.

Objectives: The primary objective of this thesis was to determine the feasibility of a translational research study (Immunometabolic CONnections to Scoliosis (ICONS) study) procedures. Secondary objectives include the performance of exploratory analyses of macrophages in PM of AIS patients on both sides of the spine. As adiposity is associated with muscle inflammation in the general population, the association of whole-body adiposity with PM macrophage content and/or phenotype was investigated.

Hypothesis: We tested the hypothesis that ICONS study protocols are feasible and that PM macrophage populations are different on both sides of the spine. Furthermore, we hypothesized that increasing adiposity positively correlates with PM macrophage infiltration.

Results: We observed that all pre-set feasibility criteria were achieved or surpassed, except that of recruitment rate. PM total macrophage content was not different on either side of the scoliotic curve; however, a trend of predominance of anti-inflammatory macrophages on the convex side of the scoliotic curve was noted. PM macrophage content correlated positively with adiposity.

Conclusions: Adopting the ICONS study procedures for the full study is feasible. Continued investigations of immune system role in PM of AIS patients may identify therapeutic targets to aid AIS treatment and prevention.

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List of Abbreviations

- %BF = Body Fat Percentage
- ΔΔCt = Delta-delta Cycle Threshold
- AIS = Adolescent Idiopathic Scoliosis
- ANOVA = Analysis of Variance
- AT = Adipose Tissue
- BCA = Bicinchoninic Acid Assay
- BMI = Body Mass Index
- bpm = Beats Per Minute
- CCL2 = Chemokine (C-C motif) Ligand 2
- CD = Cluster of Differentiation
- cDNA = Complimentary Deoxyribonucleic Acid
- ECM = Extracellular Matrix
- FAP = Fibro/Adipogenic Progenitor
- GAPDH = Glyceraldehyde 3-Phosphate Dehydrogenase
- HRP = Horseradish Peroxidase
- ICONS = Immunometabolic CONnections to Scoliosis
- IFN-γ = Interferon Gamma
- IHC = Immunohistochemistry
- IL = Interleukin
- M1 = Pro-inflammatory macrophage
- M2 = Anti-inflammatory (resident) macrophage

- mmHg = Millimeters of Mercury
- MMP = Matrix Metalloproteinase
- mRNA = Messenger Ribonucleic Acid
- PAGE = Polyacrylamide Gel Electrophoresis
- PM = Paraspinal Muscle
- PVDF = Polyvinylidene Fuoride
- qRT-PCR = Quantitative Real-Time Polymerase Chain Reaction
- RNA = Ribonucleic Acid
- SD = Standard Deviation
- SDS = Sodium Dodecyl Sulphate
- TBS-T = Tris-Buffered Saline with Tween™ 20
- TNF-α = Tumor Necrosis Factor alpha
- WHR = Waist-to-Hip Ratio
- WHtR = Waist-to-Height Ratio

Declaration of Academic Achievement

I have completed all the design, implementation and analysis of experimental work necessary to produce Figures 3-6b. Ishan Aditya and Nicola Sahar helped with participant recruitment and sample collection for Figures 1 and 2, and both are listed as co-authors in the paper resulting from aim 1 of this thesis.

CHAPTER 1:

Introduction & Statement of Problem

Adolescent Idiopathic Scoliosis: A common pediatric condition

Derived from the Greek word *skolios*, a bending, the term '*scoliosis'* describes a 3-dimensional deformity of the spine[1]. Scoliosis is primarily characterized by an abnormal curve in the coronal plane[2]. Defects in the sagittal and transverse planes include kyphosis/lordosis and vertebral rotation, respectively[3].

There are multiple causes of scoliosis including congenital abnormalities of the spine, neuromuscular abnormalities, syndromic and muscle pathology affecting muscle groups that act as spine stabilizers. Cases that have no defined etiology are labeled as Idiopathic[1].

Idiopathic scoliosis is classified into three groups depending on the age at diagnosis. Infantile (ages 0-3), juvenile (ages 4-10) and adolescent (ages 11-17) idiopathic scoliosis[4]. The most common diagnosis is Adolescent Idiopathic Scoliosis (AIS) affecting approximately 3% of children between 11 and 17 years old[1, 4-6]. While AIS is six-fold higher in females compared to males, there is yet no clear explanation for this difference[7]. To explain the dominance of AIS in females, researchers have investigated genetic arguments of X-chromosome-linked inheritance and sex hormone signaling/receptor pathologies among others, but no studies have confirmed a cause[8, 9].

Pathophysiology of AIS

Spinal curving in AIS begins from a straight spine where vertebrae rotate and unilaterally wedge for reasons still unknown [3, 10, 11]. Clinically, this is observed as a "rib-hump," or a prominent

bulge on the convex side of the spine[3, 7]. Other physical presentations include asymmetries in the shoulder and pelvic heights[7].

The severity of AIS is categorized into mild, moderate and severe groups. Severity is defined by an increasing Cobb angle, a calculation of the angle produced by the two most tilted vertebrae as seen in a postero-anterior x-ray of the spine[12].

Risk factors for progression of the scoliotic curve

Assessing the risk for curve progression is complex without a clear understanding of the mechanisms driving AIS. Clinicians use the patient's age, sex, Cobb angle and stage of skeletal maturity to estimate the likelihood of progression[1, 3, 7, 10]. Young females who are far from skeletal maturity, and presenting with curves greater than 30° are at the highest risk for progression[1, 13, 14].

Morbidities associated with AIS

Comorbidities of AIS are not common and do not usually affect mortality. Morbidities may include pulmonary hypertension, back pain, and cosmetic effects that may negatively impact self-image[7, 15-21].

AIS patients with very severe curves may present with small lung volumes due to abnormal rib cage anatomy and weak respiratory muscle strength. These abnormalities may explain the risk of right ventricular hypertrophy characteristic of pulmonary hypertension seen in autopsies of some patients with scoliosis versus those with no scoliosis[22]. Interestingly, these alterations are

rarely associated with cardiac failure and thus do not normally alter mortality rates and may go unnoticed throughout a patient's life[14, 23].

Back pain in AIS is slightly more common in adults who were untreated for AIS during their childhood years[20, 24, 25]. It is proposed that years of abnormal loading through facet joints may cause fractures in spinous processes, foramen stenosis, and posterior muscle fatigue, all of which may increase the risk of developing back pain[14, 26]. It is likely that other mechanisms are at play in AIS, however, these are not identified yet.

Several studies report poor self-image among AIS patients. This is mainly attributed to asymmetric shoulder and pelvis heights as well as a commonly observed ectomorphic body composition characterized by bony protrusions[6, 8, 27-30]. Improving self-image may be a motivator to improve treatment compliance and prevent low self-esteem, anxiety and depressive symptoms[18, 31].

Current Therapies for AIS

The *Society on Scoliosis Orthopaedic and Rehabilitation Treatment* proposes a 3-stage treatment approach for AIS[4] depending in its severity of the curve. This severity is classified by measuring the angle between the two most tilted vertebrae (Cobb angle). Clinicians recommend physiotherapy for scoliosis with a Cobb angle of 10°-25°. More severe cases, with a Cobb angle of 25°-50°, are treated with bracing, and spinal fusion surgery is used to treat curves ≥50°[32].

Observation

To ensure the early detection and to enhance treatment effectiveness, the Scoliosis Research Society recommends school screening be implemented annually[33, 34]. However, this approach is expensive, resource-intensive, and may result in an increased rate of false-positive screening results[35].

Physiotherapy

Mild cases of AIS are treated by daily torso strengthening exercise prescription and biweekly physiotherapy that includes physical manipulations and electrical muscle stimulation[3, 7, 32, 35, 36]. The basis of physical therapy is to promote structural stability to the spine via strengthening paraspinal muscles and for patients to maintain a correct posture[3, 32]. Current evidence suggests that the effectiveness of physiotherapy in altering the course of scoliosis is not conclusive[32]. Studies so far have been limited by small sample size and high risk of bias[37]. Studies are needed to assess whether exercise regimens that strengthen paraspinal muscles may lead to a reduction in curve progression [38].

Bracing

For those who do not respond to physiotherapy with curve progression to 25°, bracing is the next option of management. Depending on the location of the curve, braces can vary based on composite material and daily duration of use[3, 32]. Bracing aims to prevent respiratory dysfunction, back pain and to try and maintain an acceptable physical appearance in these patients [3, 32, 36]. The most successful bracing modality based on attenuated curve progression is the cervico-thoraco-lumbo-sacral orthosis when worn 23 hours per day [32]. Such a

demanding bracing protocol may negatively affect psychological wellbeing, albeit temporarily [39].

Surgical Correction

Approximately 11-28% of patients who use bracing and have not reached skeletal maturity will progress to a curve greater than 50°, warranting spinal instrumentation and fusion surgery[4, 40, 41]. This procedure re-aligns the spine's three-dimensional shape and aims to prevent further curve progression[32]. The risk of complications during surgery is low and the best patient-rated benefits to surgery include improved spinal appearance, self-image and psychological wellbeing among patients[42, 43]. Very few longitudinal studies have been conducted and therefore it is unclear whether surgical intervention alters the risk of scoliosis-related morbidities in adulthood[43].

Theories of AIS Etiology

Despite extensive research, the etiopathogenesis of AIS remains uncertain. Some potential contributors include genetic factors, hormones, mechanical factors, metabolic factors, the nervous system and biomechanical factors[2, 29, 35, 44-49]. Scoliosis has not been documented in other species and is likely due to their lack of bipedalism[50-52].

Genetic Factors

The exact nature of AIS onset and natural course varies greatly among patients, and family studies reveal that first-degree relatives of an AIS patient are 9% more likely to develop the disease than second-degree relatives[53]. Furthermore, twin studies indicate that concordance

rates for AIS are higher among monozygotic versus dizygotic pairs[54, 55], although this was challenged recently [56]. There are lower overall concordance rates among all twins, yet, monozygotic pairs have 25% greater risk for developing AIS[56].

Family studies suggest that AIS may be inherited, however, identifying specific genes associated remains elusive. Genome-wide association studies have recently examined single nucleotide polymorphisms that may be related to AIS development, and identified many target genes with the potential to increase AIS risk situated on chromosomes 6, 9, 16 and 17[9, 57, 58]. Furthermore, results from family studies concluded that AIS is polygenic[9, 59-64]. In summary, there appears to be a complex genetic component to AIS genesis that requires further study.

Environmental, lifestyle and Epigenetic Factors

In an attempt to understand the mechanisms driving AIS, researchers have investigated the potential influence of environmental factors on AIS genesis and prognosis via epigenetics[2, 61]. Research on sociodemographic and physical activity patterns among AIS patients is conflicting. There is some evidence suggesting that low socioeconomic status is associated with reduced physical activity participation which may augment the spine curving in AIS[65]. However, children from more affluent families who participate in dance, gymnastics and skating tend to develop a high degree of flexibility and this may contribute to AIS mechanisms by reducing spinal stability[66].

It is proposed that defects in visuospatial control of movement, caused by inactivity or hypermobility, induces repeated cycles of physical trauma to the spine and increases the

likelihood of AIS[67]. Grivas et al. (2006) proposed geographic location as a potential contributor to AIS risk. Interestingly, some northern countries have reduced yearly sunlight hours and some malnutrition among children with low sociodemographic status. This may delay puberty and attainment of skeletal maturity[68].

It is possible that factors linked to scoliotic curve progression, such as nutrition and physical activity, may alter AIS inheritance via epigenetics. Epigenetics describes the differences in phenotypes that are not linked to altered genetic sequence, but to changes in gene expression. The various modifications in gene expression can be due to methylation, histone modification and other mechanisms[2, 61, 69, 70].

Understanding AIS inheritance requires the examination of one's *Exposome* or totality of internal and external environmental exposures from conception onwards[61].

Hormones and Metabolic Factors

Pubertal AIS patients have been reported to be taller than age-matched controls after correcting for the height deformity in the coronal plane[30]. This supports the theory of abnormally accelerated vertebral bone growth as a potential contributor to AIS[71, 72]. Specifically, anterior spinal overgrowth may play an important role in causing the vertebral rotation and subsequent wedging and curving of the spine[73-75]. One suggested involve altered osteoblast function by the hormone melatonin, produced by the pineal gland[76]. Experimental studies were conducted in pinealectomized chicken as well as rats rendered bipedal via surgical amputation of the forelegs and tail[77, 78]. Interestingly, the lack of melatonin coupled with gravitational forces in

these pseudo-bipedal animals caused scoliosis[77, 78]. Further support for the potential role of melatonin in AIS is the finding that melatonin supplementation post-pinealectomy reduced scoliosis onset in chickens by 80%[77]. It is proposed that melatonin influences vertebral shape by regulating the cells involved in bone growth and remodeling[78]. Specifically, bone is formed by osteoblasts which then mature to osteocytes[79]. Another population of cells called osteoclasts is responsible for bone resorption[79]. It is believed that melatonin deficiency reduces the number of available osteocytes and lowers bone remodeling capacity, thus contributing to AIS development[76].

Melatonin is also a potent inhibitor of calmodulin, a protein responsible for shuttling Ca^{2+} ions within muscle[79]. Ca^{2+} allows myosin cross-bridges to form with actin, so reduced calmodulin levels decrease cross-bridge formation and impairs subsequent muscle contraction[79]. Alternatively, melatonin deficiency may relate to excessive calmodulin levels within paraspinal muscles causing asymmetric muscle contraction across the spine. Ultimately, this may induce a load imbalance that may initiate or perpetuate scoliosis[80]. The fundamental basis on which this and other biomechanical theories of scoliosis hinge on is that compressive and tensile forces may alter bone growth, referred to as "The Hueter-Volkmann Law." Compressive forces on epiphyseal plates may stunt bone growth while tension promotes it, contributing to deformations of the vertebral body that are associated with AIS[11, 81].

The intrinsic effects of melatonin on bone remodeling, and extrinsic calmodulin actions on the epiphyseal plates may alter vertebral growth in a manner that triggers the rotation and wedging of spinal segments.

While scoliosis models have implicated melatonin and calmodulin in scoliosis pathogenesis, so far there are no significant alterations in melatonin or calmodulin signaling in AIS patients[82]. This suggests that other mechanisms are at play in humans, or at least that these molecules may l=lay an indirect and more complex role in AIS, which may require further evaluation[82]

Nervous System & Biomechanical Factors

Nervous system dysfunction has been suggested as a potential mechanism in the development of AIS. The "asynchronous neuro-osseous growth mechanism" suggests that a short spinal cord and long anterior spine induces spinal tethering, which produces a physical stimulus inducing spinal curving as seen in AIS[44]. It has been suggested that this set of events is related to neurological factors including thinning of the cerebral cortex and a defective vestibulocochlear system characterized by impaired balance, proprioception and postural control[61, 83, 84]. Ultimately, these factors fuel a feedback loop of continued vertebral deformation until skeletal maturity is achieved and bone morphology becomes permanent.

Paraspinal Muscle Contribution to Scoliosis

Skeletal muscle is a large organ, neatly compartmentalized by layers of connective tissue. Each muscle consists of many fascicles surrounded by endomysium, and each fascicle contains groups of multi-nucleated myofibers[79]. Fascicles and myofibers are separated by connective tissue known as endomysium and perimysium, respectively[79]. Humans body have many different skeletal muscle groups, which are divided into postural and phasic types[85]. The primary role of postural muscles is to stabilize the skeleton, while phasic muscles induce movement by acting

on the skeleton[86]. The type of myofibers which compose different muscles tends to vary with functional purpose. In particular, postural muscles tend to be dominated by type I myofibers, which are capable of long bouts of low-intensity contraction[87, 88]. On the other hand, phasic muscles are dominated by type IIa and IIb myofibers, which produce short bursts of intense contraction suitable for movement[87, 88].

One example of postural muscles include the Paraspinal Muscles (PM), which are key players in maintaining balance and proprioception[85]. Muscle dysfunction in this region has been a prime suspect in AIS genesis for many years[2, 89, 90]. The success of AIS treatment is thought to depend on harnessing the torso stabilization capacity of PM. Promoting PM health might synergistically improve the effect of treatments such as physiotherapy and bracing. It may also offer the potential for preventing or slowing down spinal curving.

Past research on PM has explored electrical activity and fiber type distribution in an attempt to understand their contribution to AIS. Findings from these investigations suggest that muscle on the convex side of the scoliotic curve have a greater proportion of type 1 fibers, and exhibits slightly increased electrical activity compared to PM on the concave side of the curve[91]. It is unclear whether the alterations in muscle phenotype are adaptations or causal factors for spinal curving[92].

One potential explanation for the AIS muscle phenotype is a chronic injurious stimulus, similar to neuromuscular disorders or accidents[89, 93]. Excessive nerve root stimulation increases muscle tone and heightens the risk of structural damage at the musculotendinous junction. This

can occur due to a compensatory mechanism whereby agonist muscles over-contract and experience a faster rate of fatigue. Supporting this theory are the findings of excessive fibrosis on the concave side of the spine, suggesting that muscle injury is resulting in an asymmetrical and impaired regenerative response[90, 94]. This highlights the need to evaluate the potential role of dysfunctional paraspinal muscles at a molecular level to clarify their contribution to AIS genesis and propagation.

Normal Muscle Regeneration following injury

Injury to skeletal muscle initiates a regeneration response coordinated by the immune system. This allows satellite cells, which are muscle stem cells, to produce new myofibers[95, 96]. Resident and circulation-derived immune cells, such as macrophages and neutrophils, first sense damage-associated molecular patterns such as exposed intracellular components due to cell membrane rupture and initiate a pro-inflammatory phase. During this phase, cellular debris bind to pattern recognition receptors located on tissue-resident macrophages thus stimulating the secretion of pro-inflammatory molecules[95]. This inflammatory response is characterized by interferon gamma (INFγ), Tumour Necrosis Factor alpha (TNFα), Interleukin (IL)-1β, IL-6 and chemokine (C-C motif) Ligand 2 (CCL2) secretion among others[97].

CCL2 and other molecules create a chemoattractant signal for circulating monocytes and other immune cells to infiltrate injured muscle[97, 98]. Once monocytes enter muscle, they differentiate into inflammatory (M1) macrophages. There is a rapid increase in phagocytic immune cells which remove cellular debris, allowing for progression to the inflammation resolving/ regenerative phase[96].

Once the injury site is clear of debris, the pro-inflammatory stimulus dampens and neighbouring myofibers and macrophages secrete anti-inflammatory cytokines such as IL-10 and TGF-ß1[97]. This marks a transition towards extracellular matrix (ECM) production by fibroblasts and Fibro/Adipogenic Progenitor (FAP) cells. The purpose of ECM is to maintain tissue integrity while satellite cells differentiate and to provide scaffolding to which new myofibers may fuse. In parallel, anti-inflammatory signals induce a shift in macrophage population. This is described as alternatively activated, M2 macrophage polarization[95]. M2-macrophages stimulate fibroblast, myofibroblast and FAP cell proliferation and support satellite cell differentiation[99].

Once sufficient satellite cell differentiation has occurred, ECM is degraded by matrix metalloproteinases (MMPs) secreted my macrophages. The exact mechanism governing this final shift to phagocytic macrophages that secrete MMPs and induce FAP cell apoptosis is still unclear. Nevertheless, the final result is the restoration of muscle structure and function if this process is coordinated and terminated at the appropriate stage of healing[100, 101].

Aberrant Muscle Regeneration

During conditions of repeated muscle injury, the distinction between each of the muscle regeneration phases becomes less clear[95, 96]. Persistent danger associated molecular patterns provide pro-inflammatory stimuli, yet M2 macrophage actions lead to ECM-producing cells failure to undergo apoptosis, and there is insufficient secretion of MMPs by M1 macrophages. The final result is excessive ECM accumulation and tissue fibrosis.

Prolonged M2 activation has been shown to promote FAP cell differentiation to adipose tissue resulting in a net loss of functional myofibers[95, 96, 99, 102]. In fact, research in AIS patients has indicated that there is a marked increase in hallmark characteristics of impaired muscle regeneration, such as nuclear centralization, fibrosis and muscle atrophy, on the concave side of the spine[103]. Considering this paradigm, PM in AIS might have an asymmetric macrophage profile such that the concave side may contain more macrophages with a dominant M2 proportion.

Adiposity-driven muscle macrophage infiltration

Apart from tissue fibrosis, heightened immune activity is implicated in many diseases such as allergy/asthma, atherosclerosis, diabetes and obesity[104-106]. Since the early 1990s immune-metabolic crosstalk and its effect on tissue health has risen to the forefront of this research field. Specifically, obesity is associated with abnormal levels of inflammatory cytokines, chemical factors capable of initiating inflammatory cell signaling pathways, and this has been linked to insulin resistance in adipose tissue (AT)[107-109]. Inflammation leads to AT dysfunction and chemokine release that attract monocytes and other immune cells to AT [98, 110, 111]. Monocytes differentiate to macrophages and perpetuate an inflammatory cycle upon arrival to the tissue. AT inflammation leads to insulin resistance and will stimulate lipolysis; the spillage of fatty acids to the circulation leads to lipotoxicity and inflammation in skeletal muscle and liver[111]. Preliminary findings in murine obesity models suggest that macrophage infiltration occurs in skeletal muscle and this population is predominantly the pro-inflammatory, M1, phenotype[112-128]. Human studies of obesity driven-skeletal muscle macrophage infiltration

have been less conclusive [114, 122, 129-136]. However, the general trend is for an increase in human muscle macrophage content with obesity.

The Immunometabolic CONnections to Scoliosis (ICONS) Study

The ideal approach to addressing research questions concerning AIS etiology would involve human subjects, as there are no reliable non-human models of scoliosis. Our group has devised a protocol for a translational research project called the Immune Metabolic Connections to Scoliosis (ICONS) study[89]. In the published protocol, study procedures necessary to obtain clinical data as well as collection of muscle biopsies during AIS spinal fusion surgery were described. Furthermore, special considerations have been made to aid the investigation of how muscle inflammation and fibrosis may be involved in AIS. These considerations include the collection of PM biopsies from both sides of the spine at maximal curvature points, descriptions of experiments to assess muscle macrophage phenotype and its effect on fibrosis and FAP cell differentiation. The ICONS study is a novel undertaking involving patient data, sophisticated experimental techniques and highly technical procedures for sample collection and processing. Successful completion of this project will be contingent on appropriate resource allocation and effective collaboration between clinical and research team members. As a pre-emptive measure, and to ensure the study can answer relevant biological questions related to AIS, the ICONS study feasibility needed to be determined due to the complexity of its procedures.

Feasibility studies are detailed projects that are usually conducted in preparation of the full study[137-139]. The purpose of feasibility studies is to gain information crucial to determining whether a larger scale study will be successfully completed. Large scale studies often fail due to

complications in executing study procedures, lack of sufficient funds or time to reach study completion and inability to scientifically evaluate collected data[139]. Feasibility studies provide researchers the opportunity to critically evaluate their methods, which are expected to be applied in a larger trial and to make necessary changes without misallocating valuable resources[137, 139].

The concept of feasibility studies is commonly mistaken to that of pilot studies, that are to be used for sample size calculation and estimations of treatment effects[139]. Examples of feasibility criteria include: participant recruitment potential, ability to adhere to study protocol and completeness of data collection[140]. Evaluating feasibility criteria within the ICONS study will ensure the highest probability of the successful completion of the full study.

RESEARCH QUESTIONS

This thesis project proposes the study of the feasibility of ICONS study procedures, and to explore the role the associations of spinal curving and adiposity with inflammation in PM in children with AIS. The specific research questions are as follows:

1. Are the procedures of the ICONS study feasible?
2. Is there differential inflammation in PM muscle on both sides of the scoliotic curve in AIS patients?
3. Is PM macrophage content influenced by whole-body adiposity?

HYPOTHESES

1. In youth with AIS, conducting a translational research study of muscle Immunometabolism is feasible.
2. In AIS patients, there is an increase in M2 macrophages on the concave side of the spinal curve compared to the convex side. Furthermore, PM macrophage content is positively associated with whole-body adiposity.

AIMS

The ICONS study protocol has proposed the collection of a robust data set and biological samples from children with AIS[89]. The specific aims of this project include:

1. Determine the feasibility of the proposed ICONS study procedures.
2. Characterize macrophage content and phenotype in paraspinal muscle of AIS patients.
3. Determine the association of paraspinal muscle macrophage content and phenotype with whole-body adiposity.

CHAPTER 2: Feasibility of the ICONS study procedures.

The ICONS study is a translational research project aiming to investigate crosstalk between immune and muscle cells in AIS patients. Our goal was to evaluate the feasibility of ICONS study procedures, including the acquisition of muscle biopsies obtained from AIS patients. For this reason, the following feasibility study was completed.

Feasibility criteria are important for evaluating the potential for successfully recruiting participants, collecting samples, conducting experiments and completing data analysis. In this study, we established several feasibility criteria *a priori,* which were then used to inform the need for procedural changes to successfully complete the ICONS study.

Our results indicated that ICONS study procedures are feasible for gathering clinical and questionnaire data as well as biological samples. Furthermore, we determined that resource allocation and collaboration between research and clinical staff are also feasible regarding time management, availability of space within the hospital setting. Interestingly, we determined that the initially projected recruitment rate may not be met at our center alone due to a limited number of AIS surgeries. To ensure greater access to AIS patients undergoing spinal fusion surgery we proposed prolonging study duration as well as establishing multicenter collaborations.

Understanding muscle-immune interactions in adolescent idiopathic scoliosis: A feasibility study

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Abstract

Background: Adolescent Idiopathic Scoliosis (AIS) is the most common form of scoliosis in children, and its cause remains unknown. The Immune-metabolic CONnections to Scoliosis (ICONS) Study was designed to elucidate the potential mechanisms by which immune system-paraspinal muscle crosstalk contributes to the development of AIS. In this report, we document the evaluation of ICONS study feasibility.

Methods: This study was conducted at a tertiary pediatric academic centre in Hamilton, Ontario, Canada. We included boys and girls, aged 10-17 years with a diagnosis of AIS requiring corrective spinal surgery. Exclusion criteria included patients on high dose steroids, immunosuppressive therapy, anti-thrombotic medications, those with an active infection for 15 days before participation**,** autoimmune disease, pregnancy, non-idiopathic scoliosis and patients who were unwilling to consent.

Pre-determined feasibility criteria included permission to approach participants and recruitment rates of 80%, consenting of at least 80% of participants to provide biological samples, 90% or higher case report form and questionnaire completion, resources to be sufficient in at least 80% of recruitments, and the ability to successfully collect and process 80% or more of the biological samples needed for this study.

Results: Between August 2013-October 2014, we identified 32 potential participants with AIS, but had the resources to approach only 16, of which 12 (75%) agreed to be approached by the research team, and all consented to participate. Of the 12 participants recruited, 11 questionnaire packages and muscle biopsies (91.7% for each objective) were collected, while other biological samples (serum, plasma, whole blood for DNA and RNA processing, urine) were collected from all participants.

Conclusions: The ICONS study protocols and procedures are feasible. However, recruitment rates were less than predicted. For the full study, we plan on prolonging the recruitment phase and the inclusion of additional centers to achieve recruitment targets.

Keywords:

Adolescent Idiopathic Scoliosis

Immunometabolism

Macrophage

Paraspinal Muscle

Feasibility study

ICONS study

Strengths and limitations of this study

- Study procedures have been assessed for feasibility
- The study demonstrated the feasibility of building collaborations with clinical services essential for study conduct
- This feasibility study has informed certain modifications to the procedures implemented in the Immune-metabolic CONnections to Scoliosis (ICONS) Study. This ensures successful participant recruitment, data acquisition, and sample collection and processing.
- This feasibility study informs translational researchers of the elements that need to be considered when designing translational research studies.
- This study is potentially limited by the use of tools that rely on self-reported data, and are subject to inherent biases. However, utilizing validated tools minimized the impact of these biases.
- Another limitation is the cross-sectional nature of the study. Due to the nature of the study procedures including tissue biopsies, it is not possible to use another study design at this stage.

INTRODUCTION

Adolescent Idiopathic Scoliosis (AIS) is the most common form of spinal curving in youth, and occur mainly in girls. While the cause of AIS remains unknown, it is likely that it is caused by a complex interaction of genes, the nervous system, hormones, metabolic dysfunction, skeletal abnormalities, biochemical factors, as well as environmental and lifestyle factors[2, 29, 35, 44-49]. In some cases, AIS is associated with several comorbidities including pain, pulmonary hypertension, and cosmetic effects, which increase the burden of this condition on the affected youth [141]

Several paraspinal tissues may play a role in AIS genesis, but the exact contribution of different tissues to the genesis of AIS are not fully understood. While paraspinal muscles are important in maintaining spinal stability and mobility, it is unclear if they contribute to the initiation or propagation of spinal curvature, or if their phenotype is secondary to scoliosis. Regardless, studying muscle profile in AIS may lead to better understanding of the mechanisms involved in the development of scoliosis.

One of the important phenotypic features noted in paraspinal muscle is the infiltration of macrophages and fibrosis, which begs the question of the role of immune system-muscle interactions in AIS etiopathogenesis[90, 95, 96, 142]. While the presence of macrophages in muscle has been commented on briefly in studies that have described these findings, no detailed phenotyping of these macrophages and their contribution to muscle pathophysiology has been undertaken.

The Immune-metabolic CONnections to Scoliosis (ICONS) study was designed to investigate the mechanisms of immune-muscle crosstalk in AIS. The protocol of the study was recently

published[89]. We proposed an approach of obtaining paraspinal muscle biopsies from convex and concave sides of the scoliotic curve along with sampling of blood and urine.

The complex nature of study procedures involves consenting patients to obtain biological samples for the study, the attendance of spinal surgeries to collect tissue and other biological samples, and immediate processing of tissue samples in the operating room. It was unclear whether patients would be willing to consider participating in the study, considering the invasiveness of some of the study procedures. In addition, the feasibility of collecting high-quality biological samples needed to be determined in the clinical setting.

In addition, the final goal of the study is to recruit 120 subjects, therefore determining if timelines are realistic would be important. When completed, the ICONS study will be the largest study in the field of AIS undertaken so far, so feasibility measures are critical prior to committing resources to the full study. We did not identify any previous scoliosis studies that reported on the details of study procedures in a similar setting.

Our goal in this paper is to report on the feasibility of the ICONS study clinical processes and laboratory procedures, and indicate subsequent modifications based on these results. As studies of this nature are relatively rare in the fields of pediatric orthopaedic research and translational research, our goal was to establish the feasibility of the study procedures before launching the full study.

METHODS

Setting and Participants

The ICONS study is being conducted at McMaster Children's Hospital, a tertiary pediatric academic center in Hamilton, Ontario, Canada. In the recently published protocol, a sample size of 120 participants recruited over five years was deemed sufficient to detect significant differences in the analyses[89].

Recruitment

Patients are recruited from the Orthopedics Clinic at the Hospital. The Hamilton Integrated Research Ethics Board approved the study. Figure 1 reports study recruitment procedures.

Inclusion Criteria

We included boys and girls, 10-17 years old who are diagnosed with AIS and need corrective spinal surgery.

Exclusion Criteria

For this specific study, patients with non-idiopathic scoliosis, such as congenital scoliosis and scoliosis due to neuromuscular disorders, skeletal dysplasia and syndromes were excluded. Other reasons for exclusion included patients on high dose steroids (i.e. above the physiological substitution doses of 6-8 mg/m2/day), immunosuppressants, or anti-thrombotic medications 15 days prior to surgery. In addition, exclusion criteria included smoking, active infections, autoimmune disease, pregnancy and patients who were unable or unwilling to consent, or when anthropometric measurements were not possible[89].

Consents

The healthcare team involved in the care of potential participants obtained verbal consent from participants to be approached by the study team. If the participant and parents approve, the researcher would then introduce the study and answer their questions.

If there is agreement to participate, the youth or their parent/guardian will sign the consent forms. Participants 16 years or older signed their own consent forms, while those 10-15 years of age signed an assent form while their parent/guardian signed the consent forms. Unique identifiers were assigned to allow data anonymization shortly after data and biological sample collection to ensure confidentiality.

Collection of participant data

Standardized tools were utilized to collect data regarding nutrition[143], physical activity[144], sleep[145], mental health[146, 147], and neighbourhood walkability[148]. In addition, sociodemographic data including age, sex, ethnicity, parental and participants' education, parental profession, family income, social and family history, past medical and surgical history, current medications, pregnancy and birth history, and menstrual history in girls were collected. Anthropometric and clinical measures collected included height (cm), weight (kg), from which Body Mass Index (BMI, kg/m2) and BMI percentile calculations were derived. Waist circumference (cm), hip circumference (cm), blood pressure (mmHg), heart rate (beats per minute) and grip strength (kg) using a dynamometer were measured. The fat mass percentage was tested using bioelectrical impedance (Tanita Corporation, Arlington Heights, USA)[60]. Data were managed using Research Electronic Data Capture (REDCap) tools hosted at McMaster University [149].

Biological sample collection & processing

The biological sample processing protocol is presented in Figure 2, and reported in detail in the published study protocol[89]. Liquid biopsies including blood and urine samples were collected after a midnight fast from central lines and urinary catheters in the operating room, respectively. All samples were processed and stored within two hours of collection. Part of the muscle biopsies was snap-frozen in liquid nitrogen, and another part was preserved in formalin immediately after collection. The formalin-treated samples were then embedded in paraffin and stored until further processing.

Blood samples

Serum: Fasting serum samples were isolated from blood samples collected into anticoagulant-free tubes (BD Life Sciences, Ontario, Canada). The samples were centrifuged for 15 min at 1500g, at room temperature. Samples were aliquoted and stored at -80°C until further processing.

Plasma: Fasting plasma samples were obtained from EDTA-treated blood samples by centrifuging for 15 min at room temperature at 1500g. Samples were loaded into cryovials for storage at -80°C until further processing.

RNA samples: PAXRNA tubes (BD Life Sciences, Ontario, Canada) were used to collect and preserve leucocyte RNA from whole blood. Samples were processed according to manufacturer's instructions and then aliquoted into cryovials and stored at -80°C until further use.

DNA samples: In the original protocol, we planned on using buffy coats to obtain DNA [89]. Based on challenges in standardizing this approach, an alternative approach of obtaining whole blood samples from EDTA-containing vacutainer tubes (BD Life Sciences, Ontario, Canada) was adopted. These samples were aliquoted into cryovials and samples were stored at -80°C until further processing.

Urine samples

Fasting urine samples were collected into 90 mL containers. The samples were then aliquoted into cryovials and frozen at -80°C until further processing.

Paraspinal muscle tissue biopsies and processing

Paraspinal muscle biopsies were obtained at points of maximal concavity and convexity of spinal curves. Samples were promptly separated from connective tissue and dried from blood, and then immediately divided into few pieces. These samples were then placed in cryovials and snap-frozen in liquid nitrogen immediately. Soon after, samples were stored at -80°C until further processing. The formalin-preserved samples were embedded in paraffin by the Central Pathology Laboratory at Hamilton Health Sciences. These samples will be used in the future in staining and microscopic analyses.

Two important strategies were deployed with muscle tissue collection to preserve immune cell phenotype and integrity. First, these samples were obtained from areas where no cauterization took place, and the samples were collected before injecting adrenaline into muscle. The latter procedure is used to induce vasoconstriction but is also known to alter macrophage

phenotype[150, 151]. This approach ensures the fidelity of muscle macrophage phenotype in future analyses.

Measures to determine the feasibility of study procedures

The following criteria were used to indicate the feasibility of study procedures:
Participant and parent agreement to be approached and informed about the study in 80% of cases. Of those who agree to be approached, a minimum of 80% will be consented to participate in the study. Of those who agree to participate, case report forms and questionnaires will be completed in a minimum of 90% of participants. Resource utilization including space, researchers' time, consumables, study protocol testing and laboratory apparatus use being feasible in 80% of cases. Acquisition and processing of high-quality biological samples in 80% or more of cases.

Statistical Analysis

We evaluated the feasibility of the study procedures in 10% of the total projected study sample size target. Since our target sample size is 120 participants[89], we assessed the feasibility of the study in the first 12 subjects recruited into the study.

Data are reported including the mean (SD) for continuous variables and as a number (percent) for categorical variables. Means and standard deviations were computed using SPSS version 20.0 (IBM Corp, Armonk, New York, USA). We are also reporting medians and ranges of values to provide a more comprehensive view of the participants' data, as the sample size is small.

RESULTS

Participant recruitment

The process by which patients were approached, consented and asked to provide data is outlined in Figure 1.

Participants' Demographics

Over the period from August 2013 to October 2014, while we identified 32 potential eligible participants, we had some logistical challenges in attending the clinics and ended up attending the clinic visit for only 16 of them. This was due to the fact that the students who were involved in the recruitment having scheduling conflicts and were able to only attend a limited number of visits. On one occasion, the patient was rescheduled for their clinic visit by the clinical team, and the research team was not aware of the scheduling change.

Baseline characteristics of the group are outlined in Table 1. Of the 16 subjects, 12 (75%) agreed to be approached by the research team, and all of those approached agreed to participate in the study. Four patients were excluded due to a diagnosis of kyphosis as opposed to AIS (n=1), patient had already undergone a corrective surgery and was having a revision surgery (n=1), patient had multiple sclerosis (n=1), and patient refused to meet the team due to a scheduling conflict (n=1).

Most of those recruited were female (n=11; 91.7%). Most participants were Caucasian Europeans (n=9; 75%). The average age of was 14.4±1.9 years, and the majority were diagnosed

with significant AIS (≥40° curves) that required corrective surgery. In most cases, the spinal curve was right-sided (n=10; 83%).

Feasibility of study procedures

Pre-operative recruitment visits

Recruitment procedures were completed within 20 minutes, including presenting the research study, gathering participant signatures on consent forms and completing anthropometric measures. This indicated that the study procedures are feasible in the clinical setting where these patients are being seen prior to their surgery.

Variables	Mean (SD)	Median (range)
Age (years)	14.4 (1.9)	14.4 (11.5-17.5)
BMI (kg/m2)	20.8 (3.0)	20.1 (16.6-26.1)
BMI percentile	58.0 (27.0)	59.3 (18.5-92.5)
Body fat percentage	24.1 (6.2)	23.7 (10.3-35.4)
Cobb angle (degrees)	57.1 (14.8)	56.6 (30.0-78.7)
Pulse rate (bpm)	90.5 (14.9)	88.5 (60.0-122.0)
Systolic blood pressure (mmHg)	121.4 (9.9)	119.5 (108.0-143.0)
Diastolic blood pressure (mmHg)	75.8 (8.3)	76.5 (63.0-88.0)

Table 1: Summary of participant characteristics. Abbreviations: Standard Deviation, SD; Body Mass Index, BMI; Beats Per Minute, bpm; Millimeters of Mercury, mmHg.

All 12 subjects who were approached consented to participating in the study and to providing biological samples (100%). Case report forms reporting height, weight, body fat percentage, blood pressure and resting heart rate were completed for all subjects (100%). The participants were provided with packages containing the study questionnaires and asked to take these packages home and to give back to the team on the date of surgery. Eleven subjects returned completed questionnaire packages resulting in an overall rate of 91.7% for data collection. One family did not return the package, and further contact attempts in the post-operative period while

the patient was in hospital and by phone following discharge were not successful in retrieving the package. There have been no adverse events during the conduct of the study procedures and biological sample collection.

Operating room procedures

On the morning of the surgery, the study team attended preoperative multidisciplinary team meetings that included the operating room nursing staff, Anesthesia nursing and medical staff, and Orthopaedic surgeons. The study was highlighted to the attendees who will be present in the operating room during the procedure, and specific requirements for blood, urine and tissue samples reiterated.

Regarding space utilization in the operating room, a specific location was designated for the research team while the patient was being prepared for surgery. The operating room staff set up sterile trays with the needed equipment for biopsy processing.

Once blood, urine and tissue samples were received from the clinical staff, the research staff completed the sample processing immediately as described above.

Out of all patients who consented to participate, all 12 participants provided serum, plasma, whole blood for DNA and RNA isolation, and urine. For muscle biopsy samples, 11 sets of muscle biopsies were collected by the study team (91.7%). We could not collect muscle tissue samples from one participant due to scheduling difficulties resulting in no trained researchers available to collect and process muscle samples.

Sample processing in the laboratory

Sample processing flow is demonstrated in Figure 2. This involved centrifugation of blood samples for serum and plasma isolation as well as aliquoting PAXRNA, whole blood and urine to individual cryovials. These steps were completed within 2 hours of sample collection to maintain the integrity of samples. No significant processing problems were experienced at any stage, thus blood and urine samples from all subjects were successfully collected and stored at -80°C until further processing.

DISCUSSION

The ICONS study was designed to elucidate potential immune-metabolic mechanisms underlying AIS. Here, we report on the establishment of the feasibility of clinical and laboratory procedures of the study.

Our projected recruitment target of 80% has proven to be ambitious. However, detailed study procedure monitoring including consent procedures, instruments used for data collection, questionnaire completion rates, and biological sample collection and processing met or exceeded the pre-set targets.

Study Strengths, limitations and adjustments to procedures based on initial data

This study has several strengths. The collaborations established with different clinical services to set up recruitment procedures were instrumental in assuring successful patient recruitment. The collection of muscle biopsies prior to cauterization and adrenaline injections preserved muscle

macrophage phenotype and tissue integrity, thus allowing study questions to be addressed comprehensively, and established the feasibility of the tissue sampling for the full study.

Study procedures and equipment used have proven to be highly reliable as predetermined feasibility criteria for participant consenting, sociodemographic data collection, resource utilization and sample acquisition and processing rates were all superseded. Furthermore, the validity of all measurement tools and questionnaires implemented have already been established[143, 144]. Finally, the study protocol places limited burden on the participants, as they are providing samples while undergoing surgery, so there are no added procedures to their routine care.

We achieved lower than expected rate of participation due to scheduling challenges, but a significant number of those approached agreed to participate in the study.

Based on this data, we have designated one team member to be responsible for recruitment. This person is in charge of attending clinic visits to maximize the chances of a successful recruitment, and reduce the burden on the participants by doing all study recruitment and consenting procedures during the visit.

With these adjustments, the effect of further changes will take time to demonstrate effectiveness. We initially expected to approach around 40 patients per year based on estimated prior annual numbers of scoliosis surgeries at our center, but this was not the case during the feasibility study, with up to 30% of scoliosis cases requiring surgery being non-AIS, in addition to the situations noted above [89].

Based on our estimate of approaching 40 patients in a year we predicted a five-year recruitment period would suffice. This feasibility study is instrumental in highlighting the need for a more generous timeline for recruitment. While there is merit to this approach, the delay in knowledge generated from this project can have a significant impact on its translation to interventions that benefit patients. In addition, the sustainability of the study funding is another potential caveat to this approach.

Alternatively, expanding the study to other pediatric Orthopedic centers will help achieve our target sample size of 120 subjects. We have engaged in discussions with other centers to expand recruitment and address the sample size needed for the full study. One center is quite interested in joining the study, which will improve the recruitment timelines. There are planned discussions with other centers at this point that are interested in getting more information about the study. The feasibility of this expansion needs further evaluation to ensure its feasibility.

One potential limitation of the ICONS study is its cross-sectional design, which makes investigating causal links of muscle-immune mechanisms to AIS development difficult to establish. However, there are no reliable experimental models beyond humans to study AIS[50, 152]. In addition, the potential molecules and pathways identified in this study can be validated further in cell culture and in-vivo models as dictated by the results.

This study does not incorporate a control sample to relate muscle phenotype in AIS to non-AIS subjects. The inclusion of a control group is not feasible and is not justified ethically, as this would expose healthy children or those with less severe degrees of scoliosis to potential harms of

invasive biopsy procedures. In addition, longitudinal study designs are also not appropriate, as we cannot implement multiple biopsies to measure muscle inflammation with curve progression over time. Ultimately, our cross-sectional design is the most appropriate approach.

The study is susceptible to various sources of bias. Selection bias may occur if our sample was not representative of the pediatric population with AIS by including other types of scoliosis. This is likely to be limited, as our population of pediatric patients with AIS requiring surgery constitutes a representative group of patients for the AIS population, and we consecutively approached and recruited participants to eliminate such bias.

Recall bias may also occur when participants are completing questionnaires that inquire about subjective and/or past lifestyle or family history-related factors. We attempted to minimize this bias by correlating self-reported data with clinically obtained data that was relevant to the immediate perioperative period.

Social desirability bias may also be a threat given the subjective nature of the lifestyle questionnaires. We utilized structured data collection tools that are previously reported to reflect the clinical phenotype of children accurately[143, 144, 153, 154].

Potential outcomes of our research

The novelty of the study offers significant implications to patients with AIS. Identifying potential mechanisms underlying AIS will create new knowledge that can be translated into interventions to prevent and treat AIS. This study may also guide further experimental work in cell-based and

animal models to test specific pathways that mediate AIS. Collectively, this may allow the creation of interventions that can be tested to validate their efficacy and safety.

In addition, new insights to the complications of AIS may be gleaned from this experimental work, whereby new approaches to prevent these complications may emerge from understanding the pathogenesis of AIS, ensuring improved outcomes. Importantly, our results may develop novel insights into immune-metabolic crosstalk in AIS, which may be an important aspect of the etiopathogenesis of this condition.

Our work is also of importance in the field of translational research studies in pediatrics. AIS affects millions of children around the world, and causal factors are not fully understood. As paraspinal muscle provides spinal stability and directs spinal motion, it is critical that we understand the muscle- and immune-based mechanisms that may contribute to AIS. It is also important to inform the scoliosis research community about the potential for success of studies similar to ours, and to highlight the need for early consideration of sample size achievement. In addition, this paper will be of broader importance to colleagues performing translational research to describe approaches to study procedures and anticipated outcomes.

CONCLUSION

We have determined that the protocols and procedures for the ICONS study are feasible. However, to overcome the lower than expected recruitment rates, we will consider the

prolongation of the inclusion period and the expansion of the study to new centers. We will be moving with the implementation of these protocols taking these considerations into account.

List of Abbreviations

AIS = Adolescent Idiopathic Scoliosis

ICONS = Immune-metabolic CONnections to Scoliosis

BMI = Body Mass Index

mmHg = Millimeters of Mercury

Declarations: None

Ethics Approval: The Hamilton Integrated Research Ethics Board approved the study (HiREB approval no.12-529).

Consent for publication: Not applicable: The manuscript contains no individual person's data in any form (including individual details, images or videos), so no consent to publish have been obtained from patients or their parent or legal guardian.

Availability of data: Data are available upon request sent to the corresponding author with justification.

Competing Interests: None to be declared.

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Author Contributions: MCS is the guarantor. MCS, LT, PM, DP, and BD conceived the study question. SR, PM, DP, NS, BD, IA and MCS were responsible for creating and implementing study procedures including participant recruitment, data collection, sample processing and sample storage. MCS, PM, DP, BD, LT, IA and SR designed feasibility study criteria. LT provided additional expertise on statistical methods and assisted the development of the study design. SR, NS, IA and MCS drafted the manuscript including figures and tables. All authors contributed to the manuscript by suggesting revisions, providing comments and approving the submission of the final version of the manuscript.

Acknowledgment: We thank the participants and families for taking part in the study. We also would like to thank Ms. Kuan-Wen Wang for her editorial comments.

Figures

Figure 1: ICONS study recruitment and study procedure

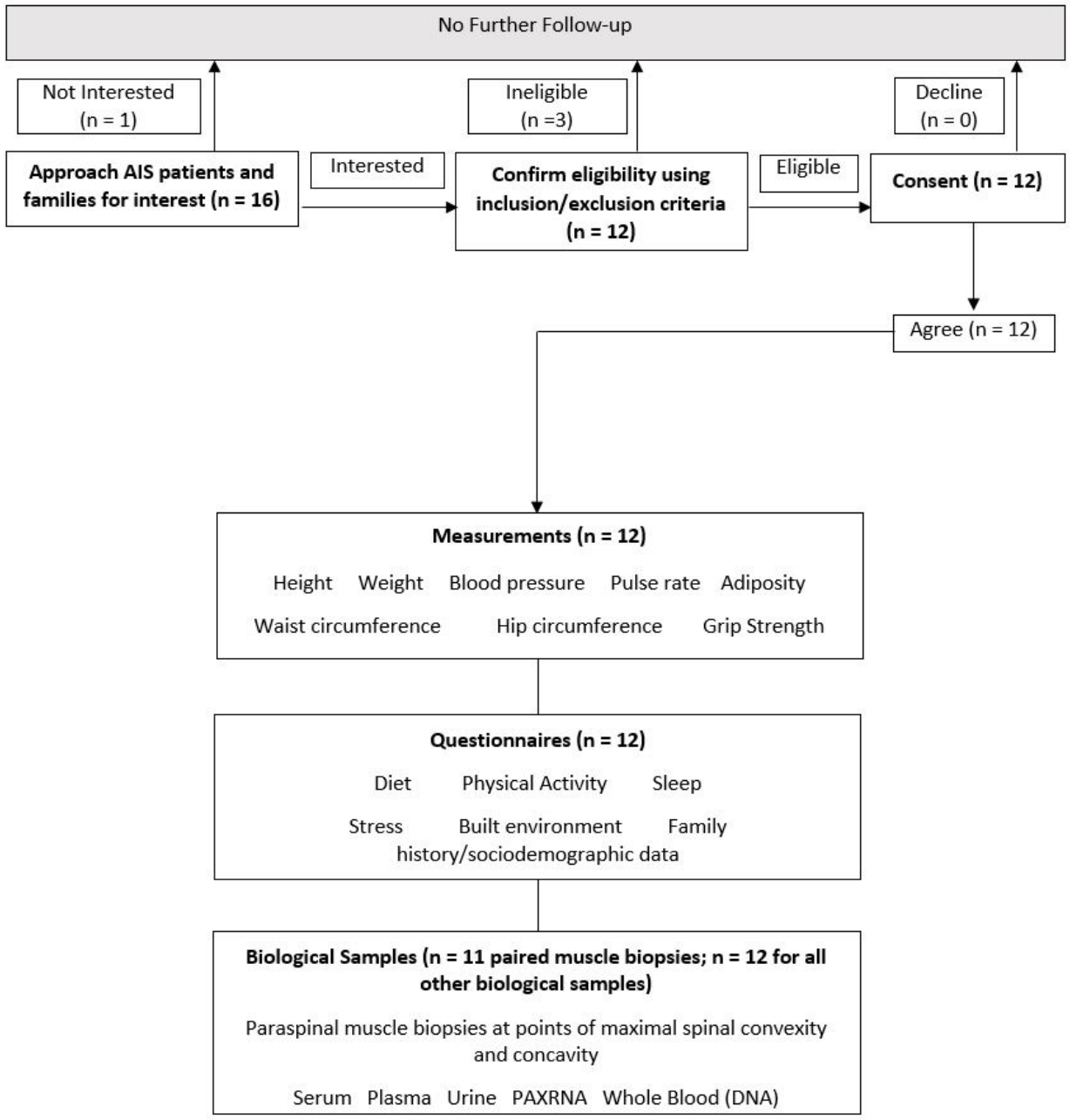
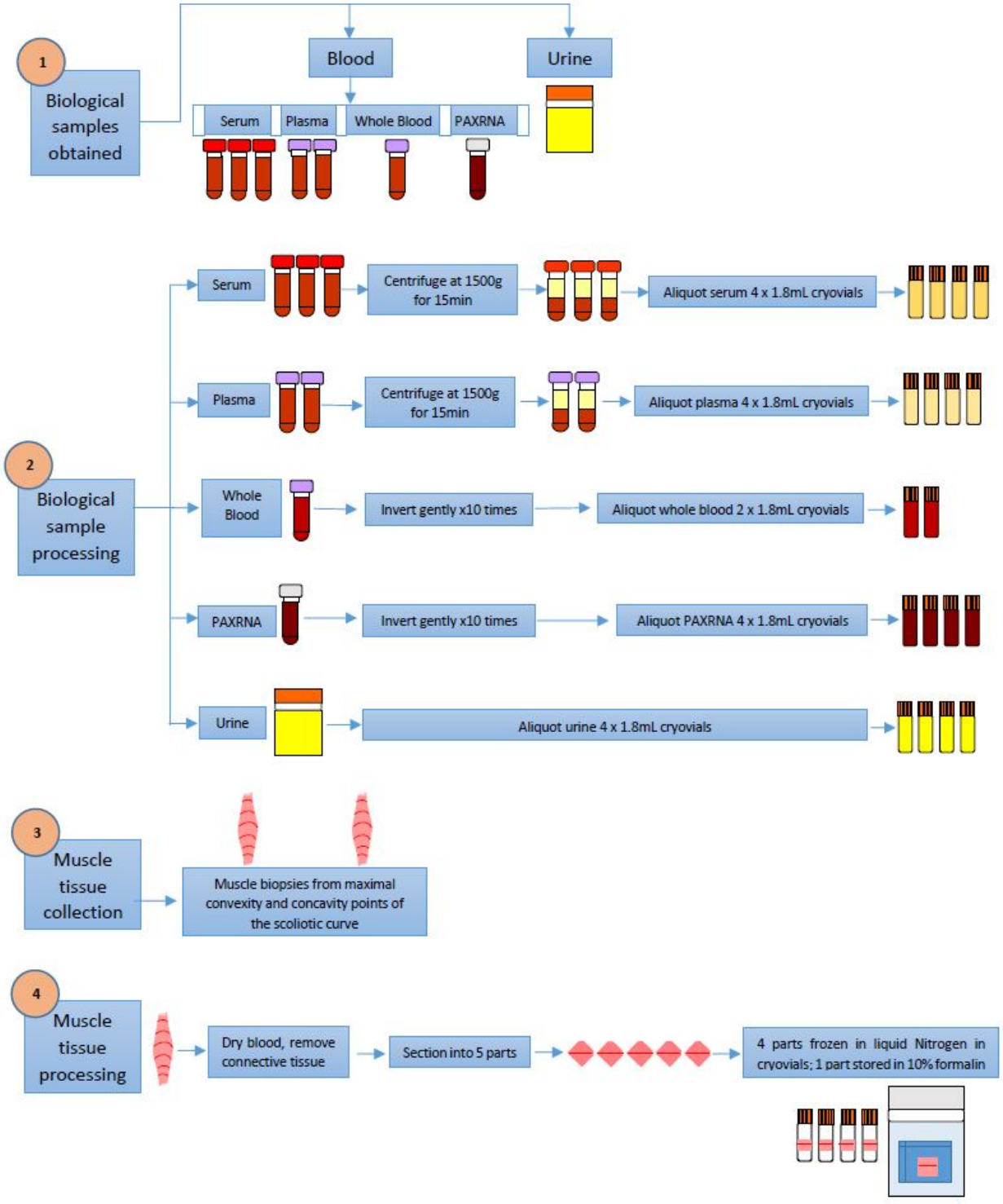


Figure 2: Biological sample processing flow chart



CHAPTER 3:

Characterization of macrophage content and phenotype in paraspinal muscle of AIS patients.

INTRODUCTION

There is a documented association between the incidence of scoliosis development and diseases that implicate dysregulated TGF-ß signaling such as Marfan syndrome (60% of patients) and Loeys-Dietz syndrome (46% of patients)[155, 156]. Thus, one proposition is that in the non-syndromic case of AIS, patients may possess an underlying genetic pathology implicating unbalanced expression of TGF-ßs and its receptors in muscles on the sides of the spine, ultimately manifesting poor PM remodeling and differential fibrosis. Elevated TGF-ß gene expression and fibrosis were observed on the concave side of the spine in AIS patients[94, 103]. The release of anti-inflammatory cytokines and growth factors is a characteristic function of M2 macrophages and is crucial to muscle remodeling by promoting satellite cell differentiation[95]. However, this M2-macrophage activity must be carefully balanced by M1-macrophage release of pro-inflammatory cytokines to induce timely ECM removal and avoid fibrosis[157]. These findings suggest that AIS patients may have dysfunctional muscle remodeling on the concave side of the spine, which may result from a persistent M2-dominant inflammatory response.

Chemokines act as chemical signals that induce monocyte migration from circulation into various tissues such as skeletal muscle[111]. For example, monocytes infiltrate skeletal muscle in response to CCL2 and later differentiate to a unique phenotype of macrophage based on the sensed microenvironment[98]. Macrophage phenotype may be defined by the presence of certain cell surface proteins. Although macrophages have been previously described as either M1 or M2, they in fact, exist on a spectrum of activated phenotypes where the extent to which M1 or M2-like characteristics are displayed is determined by the type of cell surface markers expressed, and Table 1 summarizes these patterns[158, 159]. Some of these markers include cluster of

differentiation (CD) 68, a pan-macrophage marker, CD11c (M1/dendritic cell marker) and CD206 and CD301 (M2 macrophage markers). Each of these cell surface proteins supports the macrophage's unique functions such as cytokine and growth factor recognition and cell interactions.

M1-Polarized	**M2-Polarized**
CD-11c ^{hi}	CD-11c ^{low}
CCR2 ^{hi}	CCR2 ^{low}
TLR-4 ^{hi}	TLR-4 ^{low}
TLR-2 ^{hi}	TLR-2 ^{low}
CD206 ^{low}	CD206 ^{hi}
CD301 ^{low}	CD301 ^{hi}
ARG-1 ^{low}	ARG-1 ^{hi}

Table 2: Summary of macrophage polarization markers.

METHODS

A description of participant recruitment and sample collection procedures is provided in Chapter 2 above. Further detail may also be found in the previously published protocol paper[89]. In short, patients were recruited from the orthopedic clinic at McMaster Children's Hospital. Patients who were between 10-17 years old and diagnosed with severe AIS requiring surgery were included in this study. Patients, and parents were informed of study procedures with a clear description of how anthropometric measurements and biological samples would be collected. Patients who agreed to participate, as well as their parents if under 16 years of age, signed

consent forms approved by the Hamilton Integrated Research Ethics Board. Children between 7-15 years of age signed an assent form.

Anthropometric Measurements

Once a patient agreed to participate, height and weight was measured using a stadiometer and weighing scale, respectively. These data were then used for body mass index (BMI) calculation as per the equation: BMI = Mass (Kg)/Height $(m)^2$. Furthermore, waist and hip circumference were measured using a spring-loaded measuring tape followed by body fat percent measurement using bioelectrical impedance monitor for children (Tanita Corporation, Illinois, USA).

Muscle biopsies

Erector spinae muscle samples from the apical portions of the convex and concave sides of the spine were provided obtained during surgery. It is important to note that samples were taken prior to cauterization and adrenaline injection as these measures may alter macrophage phenotype[150, 151]. Immediately following biopsy, excess blood was dried and connective tissue attachments excised from muscle. Once complete, samples were snap-frozen in liquid nitrogen and stored at -80 °C, and a small piece placed in 10% formalin until further processing[89].

Gene Expression Analysis

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) was performed on RNA isolated from muscle biopsies to quantify macrophage marker gene expression. Genes profiled included pan-macrophage marker (CD68), M1-marker (CD11c), and M2- markers (CD206, CD 301).

Homogenization was performed by suspending 50mg of powdered muscle tissue in 1000μL Trizol (Invitrogen, Mississauga) followed by bead-based mechanical homogenization at 5000rpm for 20s (Precellys Tissue Homogenizer, Bertin Instruments, Rockville, USA). The homogenate was then spun at 8000g for 10min at 4°C. RNA was then extracted and cleared of RNase contamination using 140 μL chloroform and phase separated with 600 μL of hydrophobic 100% ethanol. RNA extraction and purification was conducted with an RNeasy Mini Kit (Qiagen, Toronto) whereby, total RNA binds to the RNeasy membrane that is then washed to elute RNA back into solution.

Next, complimentary DNA (cDNA) synthesis was conducted using a SuperScript™ VILO™ cDNA Synthesis Kit (Invitrogen, Burlington, Canada). Then, 1600ng of RNA was reverse transcribed to cDNA using 2 μL of 10X SuperScript™ Enzyme Mix which includes reverse transcriptase enzyme, recombinant ribonuclease inhibitor and RNaseOUT™. In addition, 4 μL of 5X VILO™ reaction mix containing random primers, $MgCl_2$, dNTPs and an optimized buffer. The 20 μL reaction was conducted in a Thermal Cycler (Montreal Biotech Inc., Dorval, Canada) set for 25°C (10 min), 42°C (120 min) and 85°C (5 min).

Lastly, qRT-PCR of 400ng cDNA was completed in duplicate, using TaqMan assay primer/probe mixes, 10X PCR Buffer, $MgCl_2$, 2mM dNTPs (Applied Biosystems, Carlsbad, USA). Each qRT-PCR cycle consisted of 95°C for 10s and 65°C for 45s with direct detection of PCR products at 470nm using Corbett Research Rotor-Gene qRT-PCR machine (Montreal Biotech Inc., Dorval, Canada).

Protein Expression Analysis

Western blotting was conducted to quantify the macrophage marker protein content in muscle samples. We tested CD68, CD11c, CD206 and CD301. Fifty milligrams of powdered muscle tissue was combined with 300 μL of lysis buffer containing protease inhibitor (cOmplete Tablets mini, Roche, Mannheim, Germany) and 1% dithiothreitol, 5% Triton-X, 0.5% sodium orthovanadate (Sigma-Aldrich, Oakville, Canada). Mechanical homogenization was conducted as described in Gene Expression Analysis above.

To determine protein concentration, a bicinchoninic acid assay (BCA) was conducted using a Pierce™ BCA Protein Assay kit (Thermo Scientific, Rockford, USA). Following manufacturer's instructions, absorbance at 562nm for unknown proteins was extrapolated using a standard curve created by protein standards ranging from 0-2000μg/mL. Absorbance readings were conducted using a SpectraMax M5 device (Molecular Devices, (Marshall Scientific, Hampton, USA). Once concentrations of each sample were determined, aliquots of 2.5μg/μL of protein lysate were reduced using a 3:1 ratio of protein to 4X laemmli sample buffer (Bio-Rad, Mississauga, Canada). Lastly, prepared samples were boiled at 95°C for 5 minutes to ensure denaturation of proteins.

Proteins were separated based on molecular weight via sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). SDS containing polyacrylamide gels were prepared with a 4% stacking layer (0.5M Tris-HCl pH 6.8) and a 7.5% resolving layer (1.5M Tris-HCl pH 8.8). Polymerization of each layer was induced with Tetramethylethylenediamine and 10% ammonium persulfate in a 1:10 ratio. A Mini-PROTEAN Tetra Vertical Cell (Bio-Rad,

Mississauga) was used to induce a 90V electric current forcing protein to migrate through the gel for 100 minutes.

Next, a wet transfer was performed onto a polyvinylidene fluoride (PVDF) membrane. A sponge was placed on one side of a clamp. Next, blotting paper followed by PVDF membrane were placed, taking care to ensure no air bubbles were present among layers. The polyacrylamide gel containing separated protein was then gently placed on the membrane, again avoiding air bubbles. Placing another layer of blotting paper and a sponge completed this sandwich, and this was placed in a Mini-Transblot Cell (Bio-Rad, Mississauga) set at 90V current for 90 minutes at 4°C.

To visualize transfer efficiency and quantify total protein bands on the PVDF membrane, an anionic, Ponceau S stain (Sigma-Aldrich, Oakville) was applied for 5 minutes. An image of the Ponceau S stain was acquired using a Fusion Fx 7 imaging station (Montreal Biotech Incorporated, Dorval, Canada) and Vilber software version 15.11 (Fusion Marne-la-Vallée, France). Importantly, this image of the total protein was used as a loading control as opposed to housekeeping proteins, such as ß-actin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). ß-Actin have previously been shown to partake in intracellular reactions, which can alter their expression profiles[160, 161]. GAPDH levels in human skeletal muscle seem to be fiber type dependent and due to the reported uneven fiber type distribution in PM, we chose not to include a specific protein as the loading control[162]. The Ponceau S stain is an anionic stain, which binds to all proteins and thus provides an accurate estimate of protein content.

The membrane was then washed in tris-buffered saline and 1% Tween™20 (TBS-T; Sigma-Aldrich, Oakville, Canada) followed by 60-minute treatment in blocking solution of 5% non-fat milk in TBS-T. Primary rabbit, monoclonal antibodies including CD68 (Cell Signaling Technology, Beverly, USA #86985) and CD206 (Abcam, Toronto, Canada, ab64693) were applied overnight at 4°C with gentle rocking. Primary antibodies were diluted 1:1000 in 5% bovine serum albumin in TBS-T. Next, membranes were washed with TBS-T for 30 minutes and incubated with horseradish peroxidase (HRP) conjugated, anti-rabbit secondary antibodies diluted 1:10000 a solution of 5% non-fat milk in TBS-T. Bands of proteins of interest were detected using SuperSignal West Femto Maximum Sensitivity (Thermo Scientific, Mississauga, Canada), a substrate which chemiluminesces upon reaction with HRP. The chemiluminescence produced was imaged using a Fusion Fx 7 imaging station (Montreal Biotech Incorporated, Dorval, Canada) and Vilber software version 15.11 (Fusion Marne-la-Vallée, France). Protein bands were quantified using Image J, version 1.50i (National Institutes of Health, Maryland) and standardized to the total protein.

STATISTICAL ANALYSES

Participant demographics and descriptive statistics are reported as mean (standard deviation) for continuous variables, or number (%) for categorical variables. Gene expression results from qRT-PCR experiments have been analyzed using the delta-delta cycle threshold (ΔΔCt) method with respect to a reference gene (ß-actin)[163]. Results from western blots are reported as a ratio of protein of interest to total protein quantified using Ponceau S stain. Paired sample t-tests were conducted for mean comparisons on data originating from the same participant. Between-subject mean comparisons were tested for statistical significance by one-way analysis of variance

(ANOVA). Due to the small gene and protein expression sample size (n = 6-8), data were Log_{10} transformed to attempt the creation of a normally distributed variable set. P-values less than 0.05 were considered significant.

RESULTS

Demographics

Participant demographics for the 7 females and 1 male included in the preliminary analysis are provided in Table 3. All participants met inclusion/exclusion criteria and consented to undergo spinal fusion surgery. Due to small sample size, the table reports the mean and median values.

Variables	Mean (SD)	Median (range)
Age (years)	13.9 (1.6)	13 (12-17)
BMI (kg/m2)	19.3 (2.6)	19.7 (15.6-23.6)
BMI percentile	50.2 (28.9)	42.5 (18.5-83.7)
Body fat percentage	22.0 (5.7)	23.4 (10.3-27.4)
Cobb angle (degrees)	61.9 (14.3)	60.1 (40.0-80.0)
Pulse rate (bpm)	94.5 (14.5)	88.5 (83.0-122.0)
Systolic blood pressure (mmHg)	116.8 (5.8)	117 (108.0-125.0)
Diastolic blood pressure (mmHg)	71.0 (8.1)	68.5 (63.0-82.0)

***Table 3*:** Summary of participant characteristics (n=8). Abbreviations: SD, Standard Deviation; BMI, Body Mass Index; bpm, Beats Per Minute; mmHg, Millimeters of Mercury.

Macrophages in PM of AIS patients on both sides of the spinal curve

To determine if macrophages are present in PM, mRNA quantification using the ΔΔCt method was conducted for CD68, a pan-macrophage marker.

First, the difference in cycle threshold (Ct) values for CD68 and ß-actin were calculated on both sides of the spine. A sample calculation for concave PM CD68 gene expression is provided below:

$$\Delta Ct_{\text{concave CD68}} = Ct_{\text{Concave CD68}} - Ct_{\text{Concave b-actin}}$$

To compare CD68 gene expression between the sides of the scoliotic spine, all Ct values were normalized to concave PM gene expression. The equation for this is as follows:

$$\text{Convex Fold Change} = 2^{-\Delta\Delta Ct}, \text{ where } \Delta\Delta Ct = \Delta Ct_{\text{Convex CD68}} - \Delta Ct_{\text{Concave CD68}}$$

The data were Log_{10} transformed to ensure normal distribution. Means comparisons using a paired sample, student t-test revealed there were no significant differences between convex and concave CD68 mRNA levels (Figure 3, n=8; $Log(Convex/Concave_{CD68\ mRNA})=0.052$, SE=0.049, p=0.33).

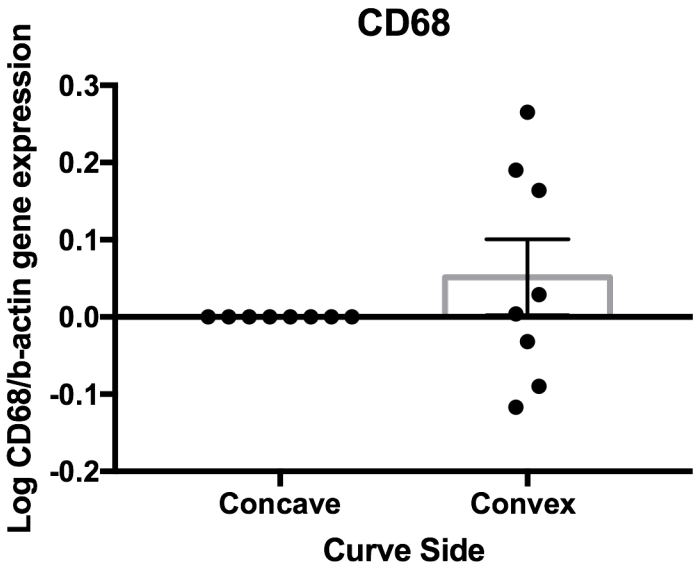


Figure 3: Convex PM CD68 gene expression normalized to concave PM; n=8

PM Macrophage phenotypic differences in PM in AIS patients

Similar calculations were conducted to evaluate the change in M1 marker, CD11c and M2-macrophage subtype markers CD206 and CD301 on the convex versus concave side. Gene expression of these macrophage subtype markers was calculated with respect to the pan-macrophage marker, CD68. A sample calculation for CD206 gene expression is provided below:

1. $\Delta Ct_{\text{concave CD206/CD68}} = Ct_{\text{Concave CD206}} - Ct_{\text{Concave CD68}}$
2. $\Delta Ct_{\text{convex CD206/CD68}} = Ct_{\text{Convex CD206}} - Ct_{\text{Concvex CD68}}$
3. Convex Fold Change = $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = \Delta Ct_{\text{Convex CD206/CD68}} - \Delta Ct_{\text{Concave CD206/CD68}}$

Again, data were Log_{10} transformed to ensure normal distribution. Next, we conducted a paired-samples, student t-test and determined that markers of macrophage subtype do not differ between the sides of the scoliotic spine (Figure 4a-c; p=0.856, 0.340 and 0.202, respectively)

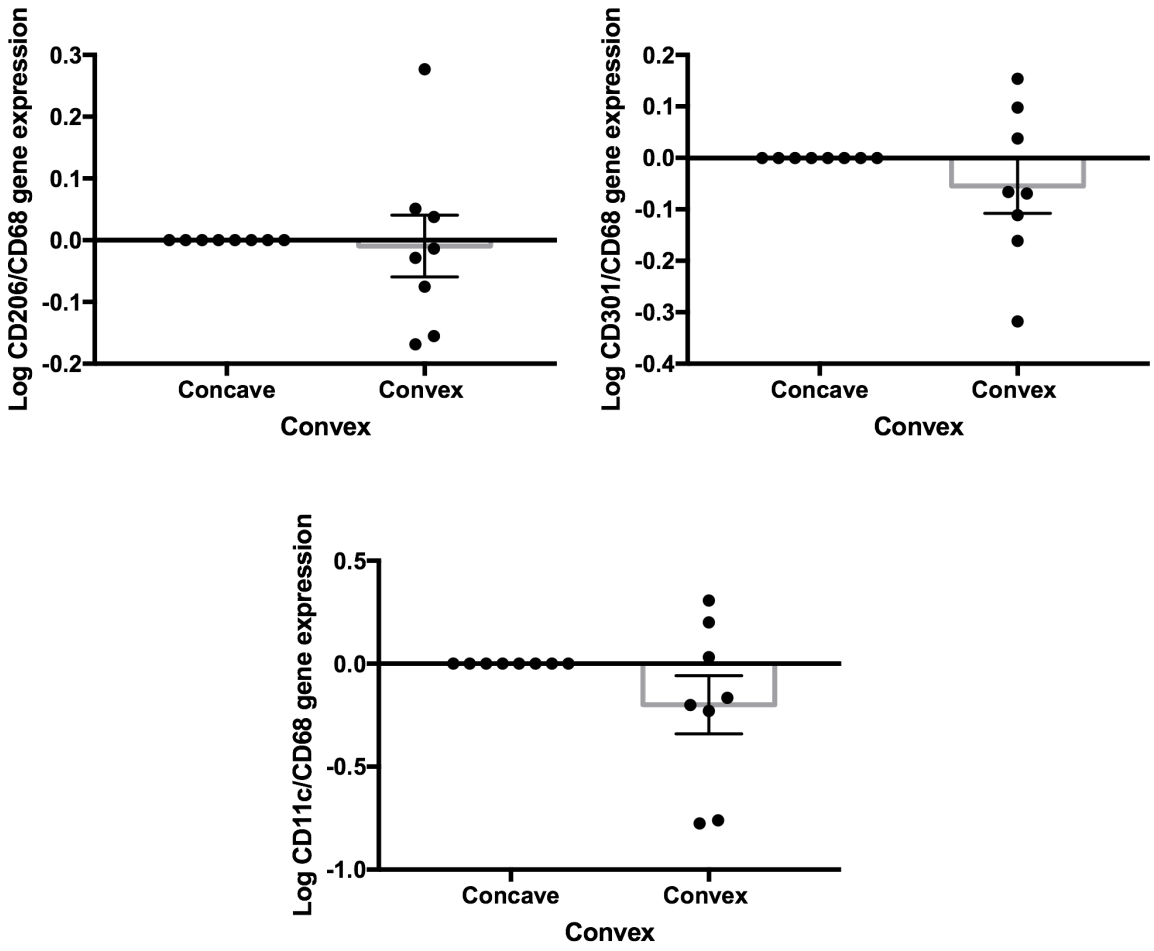


Figure 4a-c: Macrophage subtype marker gene expression with respect to pan-macrophage marker, CD68. Data are normalized to concave PM and log transformed to ensure normal distribution, n=8; a) CD206; p=0.856 b) CD301; p=0.340 c) CD11c; p=0.202.

Profiles of macrophage markers' protein expression

In accordance with the gene expression profile, western blot data indicates that CD68 protein levels are equal between the points of maximal concavity and convexity (n=6, p=0.314). Interestingly, western blot data revealed a trend towards increased M2 macrophage marker, CD206, on the convex side of the spine (n=6, p=0.072). Representative images for western blot data and a graph comparing relative CD206 expression is provided in **Figure 5**, below. CD 301 and CD11c analysis did not result in a detectable product.

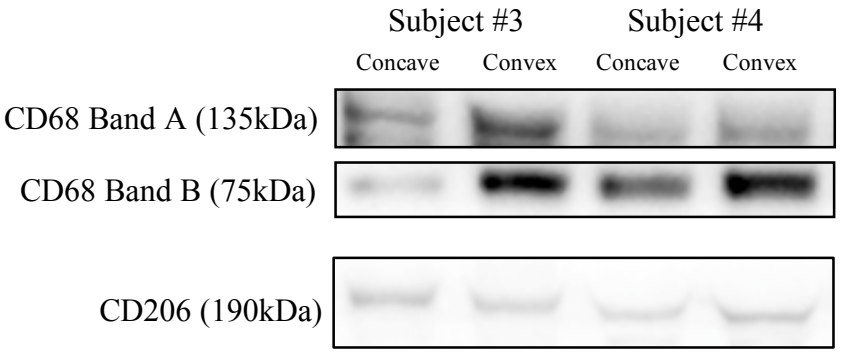


Figure 5: Representative western blot images for subjects 3 and 4 in the ICONS study.

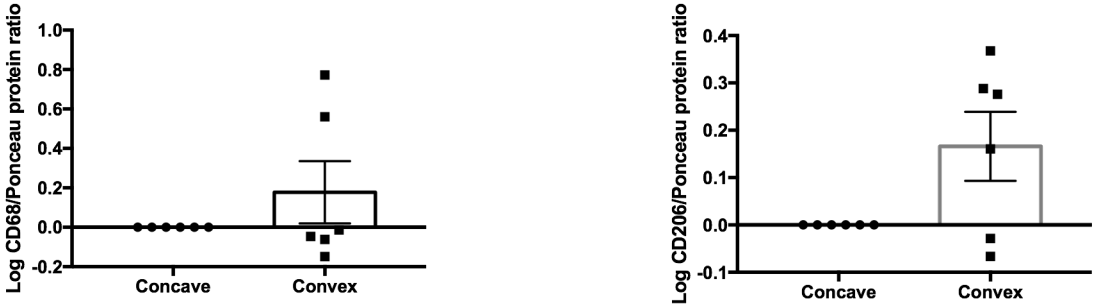


Figure 6a-b: Graphical representation of a) CD68 b) CD206 protein expression relative to total protein. n=6

DISCUSSION

We aimed to determine the content and phenotype of macrophages within both sides of the scoliotic spine PM, as we had hypothesized that concave PM would exhibit an elevated, M2-dominant macrophage population[94, 103].

To accomplish this, we conducted gene expression analyses of a pan-macrophage marker (CD68) and subtype-specific markers (CD206, CD301 and CD11c). These results suggest that, although macrophages are present in PM, their content and phenotypic distribution is not significantly different on either side of the spine.

It is important to note, gene transcripts are not biological effectors as they must still be translated to the functional protein[164]. Therefore, we conducted western blot analyses for protein expression. Accordingly, CD68 protein expression did not vary on either side. On the other hand, CD206 protein levels tended to be greater in convex PM, seemingly contradicting our hypothesis. Considering TGF-ß1 is a potent stimulator of ECM production and that macrophages are the primary source of TGF-ß1 in muscle remodeling[94, 97, 142], we hypothesized that concave PM would exhibit a dominant, M2-biased macrophage population. To better understand our results, it is important to recognize that genetic transcripts and their translational products, proteins, may not always correlate. Future studies must include a larger sample size to confirm these results.

Correlation between Macrophage Marker mRNA and Protein levels

It is thought that the production of functional proteins begins with DNA transcription to mRNA followed by its translation to a large sequence of amino acids[79]. This sequence of amino acids is then post-translationally modified to obtain its final, quaternary protein structure. With the many stages involved between mRNA production and the presence of a final protein product, we expected a degree of variability between mRNA and protein expression, which was seen with the lack of a detectable protein product for CD11c and CD301 in this small sample[164]. In fact, various post-transcriptional factors alter the concordance between qRT-PCR and western blot data by manipulating, mRNA translation rates, mRNA half-lives and intracellular location of transcripts[165-167].

Owing to its cross-sectional design, the present study cannot be used to investigate the potentially transient nature of these factors influencing mRNA translation. It has been previously suggested that patients with AIS undergo a period, approximately 18 months, when the spine begins curving and cannot be detected clinically[52]. After this period it is possible that the original curving stimulus is lost and the perpetual spinal curving occurs solely due to biomechanical reasons, thus explaining our lack of consistent findings among severe cases[52]. It is possible that during these 18 months, AIS PM experienced a period of monocyte infiltration which has since reached homeostasis. During the initial stages, these monocytes would have required stimuli to promote the production of various cell surface proteins and cytokines as part of their differentiation process[95, 157]. Since all patients included in this study presented with severe scoliosis, we may have missed this active monocyte differentiation phase thereby explaining low, and unchanged, levels of gene transcripts, which differs from that of protein expression.

Future Directions

Macrophage subsets, especially concerning the M2 phenotypes, are highly variable and our ability to identify and distinguish them is still an emerging field in scientific research. To further characterize the phenotypic distribution of macrophages in AIS PM, future studies should incorporate cytokines and growth factors uniquely produced by macrophage subtypes such as TNF-α and CCL2 by M1 and IL-4, IL-13 and TGF-ß by M2 macrophages[168].

In addition, future studies should aim to conduct Immunohistochemical (IHC) staining to co-localize various combinations of macrophage markers. This investigation would be crucial in

determining the characteristics of a unique, AIS-associated, macrophage phenotype. For example, Tidball (2017) suggests a "transitional macrophage" phenotype equally expresses M1 and M2-like characteristics and this exacerbates fibrogenic tissue formation by preventing an inflammation resolution phase[97]. IHC analyses would be valuable in determining the spatial relationship between unique types of macrophages and other objects in the tissue microenvironment. This will add further insight on the mechanisms leading to poor PM health, which is observed in AIS patients. Ultimately, a clearer understanding of a potentially dysregulated immune response to muscle regeneration may open avenues for implementing immune-based therapies to supplement the current AIS treatment options.

Chapter 4:

Determining the association of whole-body adiposity with PM macrophage content and phenotype

Introduction

Profound changes to lifestyle, including high caloric diet and sedentary lifestyles, have resulted in a global obesity epidemic[169]. In children, obesity has an especially devastating impact on longevity as this is associated with higher rates of type 2 diabetes and cardiovascular disease during adulthood[170, 171]. These metabolic diseases shorten the lifespan and increase healthcare costs drastically. To prevent these negative effects, a primary focus of medical science research today involves determining exact mechanisms by which obesity leads to metabolic diseases.

Total adiposity is measured via whole-body densitometry or bioelectrical impedance provide a rating of percent body fat (%BF), while central adiposity is measured by calculating waist-to-hip (WHR) and waist-to-height (WHtR) ratios[172]. Based on our data indicating the presence of macrophages in PM of AIS patients, we aimed to investigate whether adiposity profiles correlated with PM macrophage content and phenotype. Furthermore, considering many AIS patients present with low-fat mass, and taller stature, we were curious to determine whether this adiposity pattern correlated with a unique macrophage subtype in PM, and to see if adiposity correlates positively with inflammation in PM.

Methods

A detailed description of participant recruitment, sample collection and experimental procedures may be found in Chapters 2 and 3 above. Briefly, patients diagnosed with severe AIS and would be undergoing spinal surgery were approached. During a recruitment visit, researchers informed the patient and their family members of the study's purpose and what methods would be

involved. After detailing the patients on how body measurements and biological samples would be collected, researchers asked for their consent. If the patient was under the age of 16, the parents/ guardians would sign consent forms as well. Children 7-15 years of age signed assent forms.

Once patients signed the consent form height, weight, hip circumference and waist circumference were recorded. In addition, %BF was measured using a bioelectrical impedance scale (Tanita Corporation, Illinois, USA).

During surgery, the surgeon harvested muscle biopsies from both sides of the spine at the level of maximal curvature. Samples were then divided into smaller pieces, all of which were snap-frozen in liquid nitrogen and stored at -80°C until further processing; a small piece was preserved in 10% formalin.

Statistical Analysis

All data were log transformed before conducting the Spearman's rank correlation analysis. Data are reported such that a p-value of < 0.05 indicate statistical significance.

Results

Associations between PM macrophage content and adiposity was determined using Spearman's rank correlations. Total PM mRNA (n=8) and protein expression (n=6) of macrophage marker, CD68, was determined with respect to the adiposity descriptors, %BF, WHR and WHtR. As there were no differences in total macrophage content in PM muscle on both sides of the curve,

total PM CD68 gene expression was calculated as an average of concave and convex mRNA levels. Similarly, total PM CD68 protein expression was calculated as the average of concave and convex protein expression. Spearman rank correlation coefficients and their respective significance values are indicated in Table 4.

Total PM CD68 protein content positively correlates with fat mass

A significant, positive relationship was observed between CD68 protein and %BF (rho=0.943, p=0.005, n=6). The same trend was not seen with CD68 gene expression (rho= -0.262, p=0.531, n=8).

Correlation between total PM macrophage subtypes and adiposity

To test whether macrophage phenotype correlates with adiposity patterns, similar Spearman rank correlation analysis was conducted on protein and gene expression of the M2-macrophage marker, CD206 and M1/ dendritic cell maker, CD11c. Western blot analyses of CD11c (ab52632 1° antibody, Abcam, Toronto, Canada) yielded no protein bands. Our results indicate that neither mRNA nor protein levels of CD206 and mRNA of CD11c correlate with %BF, WHR or WHtR. Furthermore, we included analyses for the gene expression of another M2 maker, CD301 and observed a trend towards positive correlation with %BF (rho=0.707, p=0.050, n=8).

Discussion

Based on previous evidence of obesity-driven adipose tissue and skeletal muscle macrophage infiltration, we aimed to investigate whether adiposity patterns explain macrophage content in

AIS PM. To accomplish this, we determined correlations between adiposity markers (%BF, WHR and WHtR) and macrophage markers (CD68, CD206, CD301 and CD11c).

Macrophage Marker	Adiposity Measure	Spearman Rho	P-Value
CD68 Protein n=6	**% BF**	**0.943**	**0.005****
	WHR	0.257	0.623
	WHtR	0.029	0.957
CD68 mRNA n=8	% BF	-0.262	0.531
	WHR	-0.357	0.385
	WHtR	-0.405	0.320
CD206 Protein n=6	% BF	-0.086	0.872
	WHR	-0.543	0.266
	WHtR	-0.543	0.266
CD206 mRNA n=8	% BF	-0.095	0.823
	WHR	-0.167	0.693
	WHtR	-0.262	0.531
CD301 mRNA n=8	**% BF**	**0.707**	**0.050***
	WHR	0.587	0.126
	WHtR	0.491	0.217
CD11c mRNA n=8	% BF	-0.333	0.420
	WHR	0.167	0.693
	WHtR	0.167	0.693

Table 4: Table representing Spearman's Rank Correlation coefficients between PM expression of macrophage markers and adiposity measures.
*represents a trend towards statistical significance, **statistical significance ($p<0.05$)
BMI, body mass index; *WHR*, waist to hip ratio; *WHtR*, waist to height ratio

The exploratory findings of the present study indicate that a general increase in PM macrophage content correlates with increased whole-body adiposity. Furthermore, we observed a trend towards a positive correlation between CD301 mRNA levels and %BF. As suggested by Zeyda et al. (2007), this would support the theory that M2-macrophages are predominant in human tissues with an excessive lipid content[173]. The observed M2 dominance in human tissues may

be due to a compensatory mechanism preventing lipotoxicity by promoting macrophage-mediated lipid endocytosis and upregulated adipogenesis[173, 174]. Interestingly, this is not supported by CD206 gene and protein expression from the present study, suggesting PM macrophage phenotype is likely more diverse. However, larger sample size and co-staining of macrophages to discover intermediate phenotypes versus M1 and M2 phenotypes is needed to assess this.

An emerging field in Immunometabolism and obesity research is the study of infiltrating macrophages in skeletal muscle[111]. It is hypothesized that macrophages accumulate in skeletal muscle of obese individuals, which activates inflammatory signaling pathways[111, 112, 175-177]. Evidence for this hypothesis is seen in diet-induced obesity murine models[114, 115, 117, 118, 121-123, 125]. In humans however, findings are much less consistent where some indicate elevation of pro-inflammatory macrophages[114, 117, 122, 132, 136] while others indicate no change in M1-macrophage content with increasing adiposity[131, 134, 178]. Perhaps the discordance between murine and human models is due to a capacity of human physiology to buffer disturbances in tissue homeostasis[179]. Further study in human subjects is necessary to confidently determine macrophage phenotype in PM and skeletal muscle in general, and specifically among AIS patients.

Limitations and Future Directions

Our data present with discordance between protein and gene expression data, which may be a result of factors altering expression and translation of mRNA, as discussed in Chapter 3. One of these factors may be the advanced stage of scoliosis in our sample[52]. As a result, the active

inflammatory phase where gene expression directly influenced protein synthesis and thus, macrophage polarizing stimuli may have ended by the time biopsies were obtained.
Our samples may be describing the final stages whereby inflammation resolution is driving macrophage content and polarization.

Future studies should continue to characterize macrophage subtypes present in PM by means of proteomics in AIS PM. In addition, lipid accumulation in PM must be objectively quantified to further shed light on the nature of adiposity and its association with PM macrophage content. There may be differences between the postural muscles studied here compared to the past work done solely in phasic muscles such as quadriceps and gastrocnemius or soleus muscles.

Particularly, key differences in fiber type distribution may confound our findings since fiber types have unique metabolic profiles known to drive substrate utilization[180]. In fact, it has already been shown that type 1 fibers are highly oxidative and selectively metabolize lipids as a fuel source[180]. Interestingly, these fibers seem to predominate the convex muscle of PM and this is likely an adaption to the compensatory mechanisms associated with constant muscle activity that is potentially attempting to respond to the spinal curve[92, 181-183].

Furthermore, past work examining lipid accumulation has identified a phenomenon known as the "athlete's paradox" whereby endurance trained athletes exhibit increased intramuscular triglyceride accumulation[184]. Even more interestingly, skeletal muscle in these individuals does not seem to be negatively affected by the commonly associated inflammation and negative

effects on insulin signaling seen in obese individuals[184-186]. This would open more research avenues in PM of AIS patients with potential implications in the study of metabolic health.

Chapter 5: Conclusions

In an effort to assess resource allocation, and to troubleshoot the ICONS project early in its inception, the feasibility study was conducted. Subsequently, processes and procedures were deemed feasible, and will lead to project success [137-139][89].

The results of the feasibility study indicate that we achieved a lower than expected recruitment rate of 75% as opposed to 80%. However, detailed study procedure monitoring including consent procedures, data collection instruments, questionnaire completion rates, and biological sample collection and processing met or exceeded the pre-set targets. This indicates that effective collaborations with clinical and research teams can be established as part of a translational research effort. Furthermore, the quality of samples obtained is suitable for bench research to provide unparalleled insights into the mechanisms involved in AIS.

One limitation in this study was the less than predicted rate of study enrollment. To address this issue, we aim to incorporate multiple centers to ensure an adequate sample size is obtained in a timely manner. We will also aim to prolong recruitment duration.

Overall, we hope this feasibility study will inform future researchers of the optimal clinical and laboratory procedures for research on pediatric populations.

This work also demonstrated that macrophage content and phenotype appear to be similar in PM on both sides of the scoliotic curves. Larger studies are needed to confirm this trend, and to determine the presence of AIS subtypes where inflammation may be a more relevant mechanism compared to others. In addition, further analysis of macrophage phenotype and cells involved in

fibrosis e.g.FAPs, will shed light on the factors driving muscle health in AIS and will potentially help define therapies that can stop or slow down spinal curve progression and maintain PM health.

In conclusion, this work demonstrates that complex translational studies involving children are possible, and provide critical insights into common diseases in children. The knowledge gained from this work informs more advanced frameworks to study childhood scoliosis and define novel pathways that can be targeted to treat and prevent AIS.

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Appendix

Published Copy: Understanding muscle-immune interactions in adolescent idiopathic scoliosis: A feasibility study

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Pilot and Feasibility Studies

RESEARCH Open Access

Understanding muscle-immune interactions in adolescent idiopathic scoliosis: a feasibility study

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Abstract

Background: Adolescent idiopathic scoliosis (AIS) is the most common form of scoliosis in children, and its cause remains unknown. The Immune-metabolic CONnections to Scoliosis (ICONS) Study was designed to elucidate the potential mechanisms by which immune system-paraspinal muscle crosstalk contributes to the development of AIS. In this report, we document the evaluation of ICONS Study feasibility.

Methods: This study was conducted at a tertiary pediatric academic center in Hamilton, Ontario, Canada. We included boys and girls, aged 10–17 years with a diagnosis of AIS requiring corrective spinal surgery. Exclusion criteria included patients on high-dose steroids, immunosuppressive therapy, anti-thrombotic medications, those with an active infection for 15 days before participation, autoimmune disease, pregnancy, and patients who were unwilling to consent.
Pre-determined feasibility criteria included permission to approach participants and recruitment rates of 80%, consenting of at least 80% of participants to provide biological samples, 90% or higher case report form and questionnaire completion, resources to be sufficient in at least 80% of recruitments, and the ability to successfully collect and process 80% or more of the biological samples needed for this study.

Results: Between August 2013 and October 2014, we identified 32 potential participants with AIS, but had the resources to approach only 16, of which 12 (75%) agreed to be approached by the research team, and all consented to participate. Of the 12 participants recruited, 11 questionnaire packages and muscle biopsies (91.7% for each objective) were collected, while other biological samples (serum, plasma, whole blood for DNA and RNA processing, urine) were collected from all participants.

Conclusions: The ICONS study protocols and procedures are feasible. However, recruitment rates were less than predicted. For the full study, we plan on prolonging the recruitment phase and the inclusion of additional centers to achieve recruitment targets.

Keywords: Adolescent idiopathic scoliosis, Immunometabolism, Macrophage, Paraspinal muscle, Feasibility study, ICONS study

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Strengths and limitations of this study

- Study procedures have been assessed for feasibility.
- The study demonstrated the feasibility of building collaborations with clinical services essential for study conduct.
- This feasibility study has informed certain modifications to the procedures implemented in the Immune-metabolic CONnections to Scoliosis (ICONS) Study. This ensures successful participant recruitment, data acquisition, and sample collection and processing.
- This feasibility study informs translational researchers of the elements that need to be considered when designing translational research studies.
- This study is potentially limited by the use of tools that rely on self-reported data, and are subject to inherent biases. However, utilizing validated tools minimized the impact of these biases.
- Another limitation is the cross-sectional nature of the study. Due to the nature of the study procedures including tissue biopsies, it is not possible to use another study design at this stage.

Background

Adolescent idiopathic scoliosis (AIS) is the most common form of spinal curving in youth, and occurs mainly in girls. While the cause of AIS remains unknown, it is likely that it is caused by a complex interaction of genes, the nervous system, hormones, metabolic dysfunction, skeletal abnormalities, biochemical factors, as well as environmental and lifestyle factors [1–9]. In some cases, AIS is associated with several comorbidities including pain, pulmonary hypertension, and cosmetic effects, which increase the burden of this condition on the affected youth although the impact of AIS on these comorbidities and the justification for surgery based on them has been debated [10].

Several paraspinal tissues may play a role in AIS, but the exact contribution of different tissues to the genesis of AIS are not fully understood. While paraspinal muscles are important in maintaining spinal stability and mobility, it is unclear if they contribute to the initiation or propagation of spinal curvature, or if their phenotype is secondary to scoliosis. Regardless, studying muscle profile in AIS may lead to better understanding of the mechanisms involved in the development of scoliosis.

One of the important phenotypic features noted in paraspinal muscle is the infiltration of macrophages and fibrosis, which begs the question of the role of immune system-muscle interactions in AIS etiopathogenesis [11–14]. While the presence of macrophages in muscle has been commented on briefly in studies that have described these findings, no detailed phenotyping of these macrophages and their contribution to muscle pathophysiology has been undertaken.

The Immune-metabolic CONnections to Scoliosis (ICONS) Study was designed to investigate the mechanisms of immune-muscle crosstalk in AIS. The protocol of the study was recently published [15].

The complex nature of study procedures involve consenting patients to obtain biological samples for the study, the attendance of spinal surgeries to collect tissue and other biological samples, and immediate processing of tissue samples in the operating room. It was unclear whether patients would be willing to consider participating in the study, considering the invasiveness of some of the study procedures. In addition, the feasibility of collecting high-quality biological samples needed to be determined in the clinical setting.

The final goal of the study is to recruit 120 subjects, therefore determining if timelines are realistic would be important. When completed, the ICONS study will be the largest study in the field of AIS immunometabolism undertaken so far, so feasibility measures are critical prior to committing resources to the full study. We did not identify any previous scoliosis studies that reported on the details of study procedures in a similar setting.

Our goal in this paper is to report on the feasibility of the ICONS study clinical processes and laboratory procedures, and indicate subsequent modifications based on these results. As studies of this nature are relatively rare in the fields of pediatric orthopedic and translational research, our goal was to establish the feasibility of the study procedures before launching the full study.

Methods

Setting and participants

The ICONS Study is being conducted at McMaster Children's Hospital, a tertiary pediatric academic center in Hamilton, Ontario, Canada. In the recently published protocol, a sample size of 120 participants recruited over 5 years was deemed sufficient to detect significant differences in the analyses [15].

Recruitment

Patients are recruited from the Orthopedics Clinic at the Hospital. The Hamilton Integrated Research Ethics Board approved the study. Figure 1 reports study recruitment procedures.

Inclusion criteria

We included boys and girls, 10–17 years old, who are diagnosed with AIS and need corrective spinal surgery.

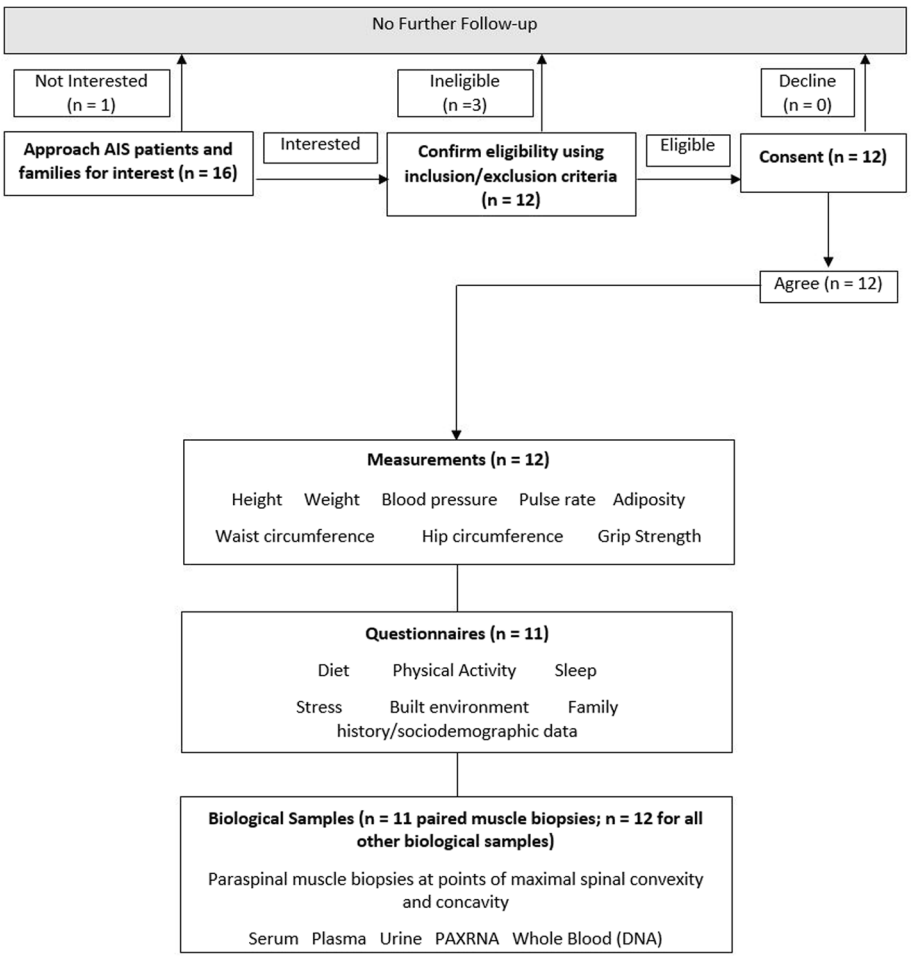

Fig. 1 ICONS Study recruitment and study procedures

Exclusion criteria

For this specific study, patients with non-idiopathic scoliosis, such as congenital scoliosis and scoliosis due to neuromuscular disorders, skeletal dysplasia, and syndromes were excluded. Other reasons for exclusion included patients on high dose steroids (i.e. above the physiological substitution doses of 6–8 mg/m^2/day), immunosuppressants, or anti-thrombotic medications 15 days prior to surgery. In addition, exclusion criteria included smoking, active infections, autoimmune disease, pregnancy, and patients who were unable or unwilling to consent, or when anthropometric measurements were not possible [15].

Consent

The healthcare team involved in the care of potential participants obtained verbal consent from participants to be approached by the study team. If the participant and parents approve, the researcher would then introduce the study and answer their questions.

If there is agreement to participate, the youth or their parent/guardian will sign the consent forms. Participants 16 years or older signed their own consent forms, while those 7–15 years of age signed an assent form while their parent/guardian signed the consent forms. Unique identifiers were assigned to allow data anonymization shortly after data and biological sample collection to ensure confidentiality.

Collection of participant data

Standardized tools were utilized to collect data regarding nutrition [16], physical activity [17], sleep [18], mental health [19, 20], and neighborhood walkability [21]. In addition, sociodemographic data including age, sex, ethnicity, parental and participants' education, parental profession, family income, social and family history, past medical and surgical history, current medications, pregnancy and birth history, and menstrual history in girls were collected. Anthropometric and clinical measures collected included height (cm), weight (kg), from which Body Mass Index (BMI, kg/m^2) and BMI percentile

calculations were derived. Waist circumference (cm), hip circumference (cm), blood pressure (mmHg), heart rate (beats per minute), and grip strength (kg) using a dynamometer were measured. The fat mass percentage was tested using bioelectrical impedance (Tanita Corporation, Arlington Heights, USA) [22]. Data were managed using Research Electronic Data Capture (REDCap) tools hosted at McMaster University [23].

Biological sample collection and processing

The biological sample processing protocol is presented in Fig. 2, and reported in detail in the published study protocol [15]. Liquid biopsies including blood and urine samples were collected after a midnight fast from central lines and urinary catheters in the operating room, respectively. All samples were processed and stored within two hours of collection. Part of the muscle biopsies was snap-frozen in liquid nitrogen, and another part was preserved in formalin immediately after collection. The formalin-treated samples were then embedded in paraffin and stored until further processing.

Blood samples

Serum: Fasting serum samples were isolated from blood samples collected into anticoagulant-free tubes (BD Life Sciences, Ontario, Canada). The samples were centrifuged for 15 min at 1500*g*, at room temperature. Samples were aliquoted and stored at −80 °C until further processing.

Plasma: Fasting plasma samples were obtained from EDTA-treated blood samples by centrifuging for 15 min at room temperature at 1500*g*. Samples were loaded into cryovials for storage at −80 °C until further processing.

RNA samples: PAXRNA tubes (BD Life Sciences, Ontario, Canada) were used to collect and preserve leucocyte RNA

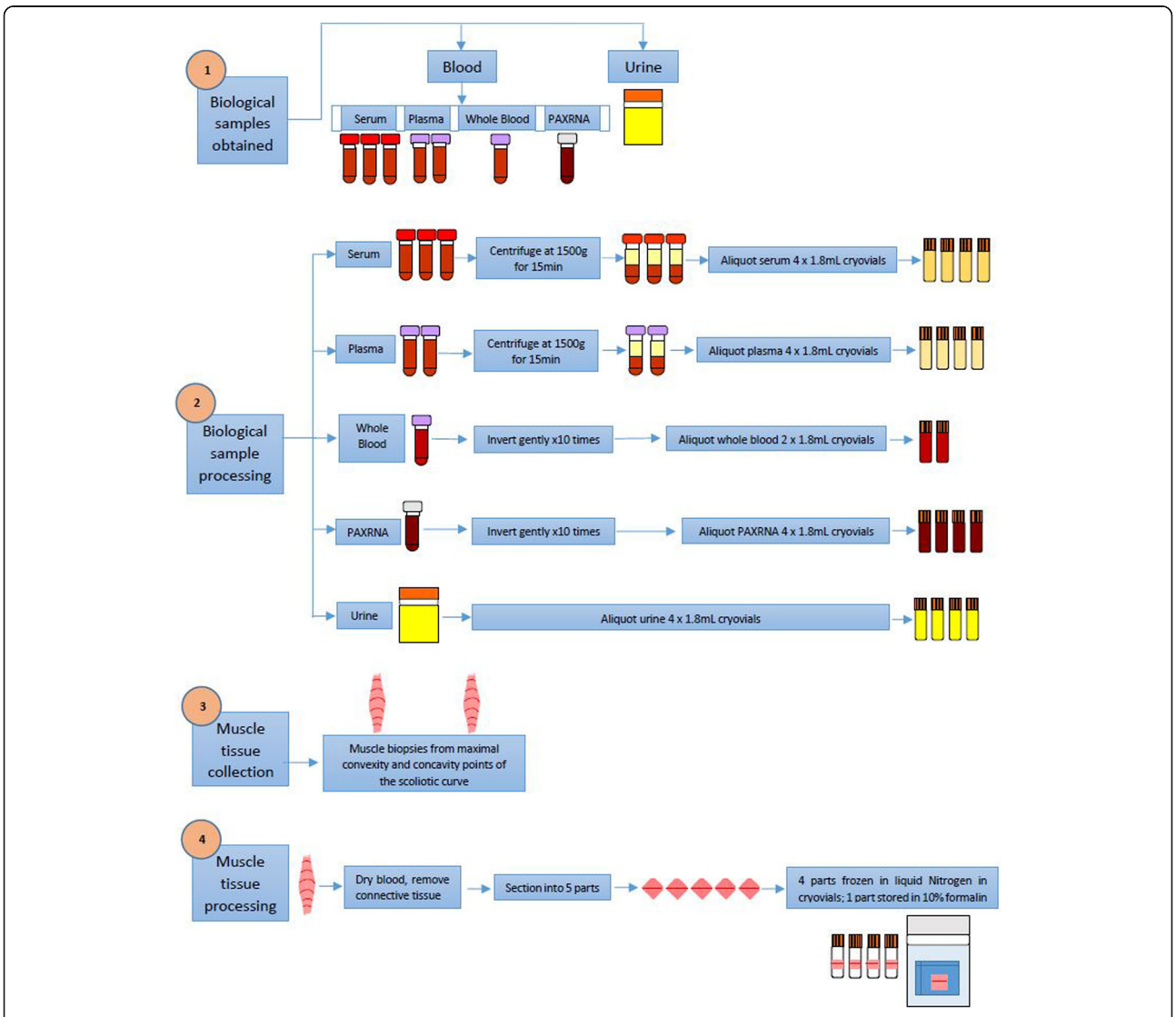

Fig. 2 Biological sample processing flow chart

from whole blood. Samples were processed according to manufacturer's instructions and then aliquoted into cryovials and stored at −80 °C until further use.

DNA samples: In the original protocol, we planned on using buffy coats to obtain DNA [15]. Based on challenges in standardizing this approach, an alternative approach of obtaining whole blood samples from EDTA-containing vacutainer tubes (BD Life Sciences, Ontario, Canada) was adopted. These samples were aliquoted into cryovials and samples were stored at −80 °C until further processing.

Urine samples

Fasting urine samples were collected into 90-mL containers. The samples were then aliquoted into cryovials and frozen at −80 °C until further processing.

Paraspinal muscle tissue biopsies and processing

Paraspinal muscle biopsies were obtained at points of maximal concavity and convexity of spinal curves. Samples were promptly separated from connective tissue and dried from blood, and then divided into few pieces. These samples were then placed in cryovials and snap-frozen in liquid nitrogen immediately. Soon after, samples were transferred in liquid nitrogen and stored at −80 °C until further processing. The formalin-preserved samples were embedded in paraffin by the Central Pathology Laboratory at Hamilton Health Sciences. These samples will be used in the future in staining and microscopic analyses.

Two important strategies were deployed with muscle tissue collection to preserve immune cell phenotype and integrity. First, these samples were obtained from areas where no cauterization took place, and the samples were collected before injecting adrenaline into muscle. The latter procedure is used to induce vasoconstriction but adrenaline is also known to alter macrophage phenotype [24, 25]. This approach ensures the fidelity of muscle macrophage phenotype in future analyses.

Measures to determine the feasibility of study procedures

The following criteria were used to indicate the feasibility of study procedures:

1. Participant and parent agreement to be approached and informed about the study in 80% of cases.
2. Of those who agree to be approached, a minimum of 80% will be consented to participate in the study.
3. Of those who agree to participate, case report forms and questionnaires will be completed in a minimum of 90% of participants.
4. Resource utilization including space, researchers' time, consumables, study protocol testing, and laboratory apparatus use being feasible in 80% of cases.
5. Acquisition and processing of high-quality biological samples in 80% or more of cases.

Statistical analysis

We evaluated the feasibility of the study procedures in 10% of the total projected study sample size target. Since our target sample size is 120 participants [15], we assessed the feasibility of the study in the first 12 subjects recruited into the study.

Data are reported including the mean (SD) for continuous variables and as a number (percent) for categorical variables. Means and standard deviations were computed using SPSS version 20.0 (IBM Corp., Armonk, New York, USA). We are also reporting medians and ranges of values to provide a more comprehensive view of the participants' data, as the sample size is small.

Results

Participant recruitment

The process by which patients were approached, consented, and asked to provide data is outlined in Fig. 1.

Participants' demographics

Over the period from August 2013 to October 2014, while we identified 32 potential eligible participants, we had some logistical challenges in attending the clinics and ended up attending the clinic visit for only 16 of them. This was due to the fact that the students who were involved in the recruitment had scheduling conflicts and were able to only attend a limited number of visits. On one occasion, the patient was rescheduled for their clinic visit by the clinical team, and the research team was not aware of the scheduling change.

Baseline characteristics of the group are outlined in Table 1. Of the 16 subjects, 12 (75%) agreed to be approached by the research team, and all of those approached agreed to participate in the study. Four patients were excluded due to a diagnosis of kyphosis as opposed to

Table 1 Summary of participant characteristics

Variables	Mean (SD)	Median (range)
Age (years)	14.4 (1.9)	14.4 (11.5–17.5)
BMI (kg/m2)	20.8 (3.0)	20.1 (16.6–26.1)
BMI percentile	58.0 (27.0)	59.3 (18.5–92.5)
Body fat percentage	24.1 (6.2)	23.7 (10.3–35.4)
Cobb angle (degrees)	57.1 (14.8)	56.6 (30.0–78.7)
Pulse rate (bpm)	90.5 (14.9)	88.5 (60.0–122.0)
Systolic blood pressure (mmHg)	121.4 (9.9)	119.5 (108.0–143.0)
Diastolic blood pressure (mmHg)	75.8 (8.3)	76.5 (63.0–88.0)

Abbreviations: *SD* standard deviation, *BMI* body mass index, *bpm* beats per minute, *mmHg* millimeters of mercury. All 12 subjects who were approached consented to participating in the study and to providing biological samples (100%)

AIS (n = 1), patient had already undergone a corrective surgery and was having a revision surgery (n = 1), patient had multiple sclerosis (n = 1), and patient refused to meet the team due to a scheduling conflict (n = 1).

Most of those recruited were female (n = 11; 91.7%). Most participants were Caucasian Europeans (n = 9; 75%). The average age of participants was 14.4 ± 1.9 years, and the majority were diagnosed with significant AIS (≥ 40° curves) that required corrective surgery. In most cases, the spinal curve was right-sided (n = 10; 83%).

Feasibility of study procedures

Pre-operative recruitment visits

Recruitment procedures were completed within 20 min, including presenting the research study, gathering participant signatures on consent forms and completing anthropometric measures during visit. This indicated that the study procedures are feasible in the clinical setting where these patients are being seen prior to their surgery.

Case report forms reporting height, weight, body fat percentage, blood pressure, and resting heart rate were completed for all subjects (100%). The participants were provided with packages containing the study questionnaires and asked to take these packages home and to return them to the team on the date of surgery. Eleven subjects returned completed questionnaire packages resulting in an overall rate of 91.7% for data collection. One family did not return the package, and further contact attempts in the post-operative period while the patient was in hospital and by phone following discharge were not successful in retrieving the package.

There have been no adverse events during the conduct of the study procedures and biological sample collection.

Operating room procedures

On the morning of the surgery, the study team attended preoperative multidisciplinary team meetings that included the operating room nursing staff, anesthesia nursing and medical staff, and orthopedic surgeons. The study was highlighted to the attendees who will be present in the operating room during the procedure, and specific requirements for blood, urine, and tissue samples reiterated.

Regarding space utilization in the operating room, a specific location was designated for the research team while patients were being prepared for surgery. The operating room staff set up sterile trays with the needed equipment for biopsy processing.

Once blood, urine, and tissue samples were received from the clinical staff, the research staff completed the sample processing immediately as described above.

Out of all patients who consented to participate, all 12 participants provided serum, plasma, whole blood for DNA and RNA isolation, and urine. For muscle biopsy samples, 11 sets of muscle biopsies were collected by the study team (91.7%). We could not collect muscle tissue samples from one participant due to scheduling difficulties resulting in no trained researchers available to collect and process muscle samples.

Sample processing in the laboratory

Sample processing flow is demonstrated in Fig. 2. This involved centrifugation of blood samples for serum and plasma isolation as well as aliquoting PAXRNA, whole blood and urine to individual cryovials. These steps were completed within two hours of sample collection to maintain the integrity of the biological samples. No significant processing problems were experienced at any stage, thus blood and urine samples from all subjects were successfully collected and stored at −80 °C until further processing.

Discussion

The ICONS Study was designed to elucidate potential immune-metabolic mechanisms underlying AIS. Here, we report on the establishment of the feasibility of clinical and laboratory procedures of the study.

This feasibility study has helped us revisit our project recruitment target of 80%. Detailed study procedure monitoring including consent procedures, instruments used for data collection, questionnaire completion rates, and biological sample collection and processing met or exceeded the pre-set targets.

Study strengths, limitations, and adjustments to procedures based on initial data

This study has several strengths. The collaborations established with different clinical services to set up recruitment procedures were instrumental in assuring successful patient recruitment. The collection of muscle biopsies prior to cauterization and adrenaline injections preserved muscle macrophage phenotype and tissue integrity, thus allowing study questions to be addressed comprehensively, and established the feasibility of tissue sampling for the full study.

Study procedures and equipment used have proven to be highly reliable as predetermined feasibility criteria for participant consenting, sociodemographic data collection, resource utilization and sample acquisition and processing rates were all superseded. Furthermore, the validity of all measurement tools and questionnaires implemented have already been established [16, 17]. Finally, the study protocol places limited burden on the participants, as they are providing samples while undergoing surgery, so there are no added procedures to their routine care.

We achieved a lower than expected rate of participation due to scheduling challenges, but a significant number of those approached agreed to participate in the study.

Based on this data, we have designated one team member to be responsible for recruitment. This person is in charge of attending clinic visits to maximize the chances of a successful recruitment, and reduce the burden on the participants by doing all study recruitment and consenting procedures during the visit.

With these adjustments, the effect of further changes will take time to demonstrate effectiveness. We initially expected to approach around 40 patients per year based on estimated prior annual numbers of scoliosis surgeries at our center, but this was not the case during the feasibility study, with up to 30% of scoliosis cases requiring surgery being not AIS, in addition to the situations noted above [15].

Based on our estimate of approaching 40 patients in a year we predicted a 5-year recruitment period would suffice. This feasibility study is instrumental in highlighting the need for more generous timelines for recruitment. While there is merit to this approach, the delay in knowledge generated from this project can have a significant impact on its translation to interventions that benefit patients. In addition, the sustainability of the study funding is another potential caveat to this approach.

Alternatively, expanding the study to other pediatric orthopedic centers will help achieve our target sample size of 120 subjects. We have engaged in discussions with other centers to expand recruitment and address the sample size needed for the full study. One center is quite interested in joining the study, which will improve the recruitment timelines. There are planned discussions with other centers that are interested in getting more information about the study. The feasibility of this expansion needs further evaluation to ensure its feasibility.

One potential limitation of the ICONS study is its cross-sectional design, which makes investigating causal links of muscle-immune mechanisms to AIS development difficult to establish. However, there are no reliable experimental models beyond humans to study AIS [26, 27]. In addition, the potential molecules and pathways identified in this study can be validated further in cell culture and in vivo models as dictated by the results.

This study does not incorporate a control sample to compare muscle phenotype in AIS to non-AIS subjects. The inclusion of a control group is neither feasible nor ethically justified, as this would expose healthy children or those with less severe degrees of scoliosis to potential harms of invasive biopsy procedures. In addition, longitudinal study designs are also not appropriate, as we cannot implement multiple biopsies to measure muscle inflammation with curve progression over time, and repeated biopsies may have their own inflammatory response in muscle at sites of biopsy. Ultimately, our cross-sectional design is the most appropriate approach.

The study is susceptible to various sources of bias. Selection bias may occur if our sample was not representative of the pediatric population with AIS by including other types of scoliosis. This is likely to be limited, as our population of pediatric patients with AIS requiring surgery constitutes a representative group of patients with AIS.

Recall bias may also occur when participants are completing questionnaires that inquire about subjective and/or past lifestyle or family history-related factors. We attempted to minimize this bias by correlating self-reported data with clinically obtained data that was relevant to the immediate perioperative period.

Social desirability bias may also be a threat given the subjective nature of the lifestyle questionnaires. We utilized structured data collection tools that are previously reported to reflect the clinical phenotype of children accurately [16, 17, 23].

Potential outcomes of our research

The novelty of the study offers significant implications to patients with AIS. Identifying potential mechanisms underlying AIS will create new knowledge that can be translated into interventions to prevent and treat AIS. This study may also guide further experimental work in cell-based and animal models to test specific pathways that mediate AIS. Collectively, this may allow the creation of interventions that can be tested to validate their efficacy and safety.

In addition, new insights to the complications of AIS may be gleaned from this experimental work, whereby new approaches to prevent these complications may emerge from understanding the pathogenesis of AIS, ensuring improved outcomes. Importantly, our results may develop novel insights into immune-metabolic crosstalk in AIS, which may be an important aspect of the etiopathogenesis of this condition.

Our work is also of importance in the field of translational research studies in pediatrics. AIS affect millions of children around the world, and causal factors are not fully understood. As paraspinal muscle provides spinal stability and directs spinal motion, it is critical that we understand the muscle- and immune-based mechanisms that may contribute to AIS. It is also important to inform the scoliosis research community about the potential for success of studies similar to ours, and to highlight the need for early consideration of sample size achievement. In addition, this paper will be of broader importance to colleagues performing translational research to describe approaches to study procedures and anticipated outcomes.

Conclusion

We have determined that the protocols and procedures for the ICONS study are feasible. However, to overcome the lower than expected recruitment rates, we will consider the prolongation of the inclusion period and the expansion of the study to new centers. We will be moving with the implementation of these protocols taking these considerations into account.

Abbreviations

AIS: Adolescent idiopathic scoliosis; BMI: Body mass index; ICONS: Immune-Metabolic CONnections to Scoliosis; mmHg: Millimeters of mercury

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Availability of data and materials

Data are available upon request sent to the corresponding author with justification.

Authors' contributions

MCS is the guarantor. MCS, LT, PM, DP, and BD conceived the study question. SR, PM, DP, NS, BD, IA, and MCS were responsible for creating and implementing study procedures including participant recruitment, data collection, sample processing, and sample storage. MCS, PM, DP, BD, LT, IA, and SR designed feasibility study criteria. LT provided additional expertise on statistical methods and assisted the development of the study design. SR, NS, IA, and MCS drafted the manuscript including figures and tables. All authors contributed to the manuscript by suggesting revisions, providing comments, and approving the submission of the final version of the manuscript.

Ethics approval and consent to participate

The Hamilton Integrated Research Ethics Board approved the study (HiREB approval no. 12–529).

Consent for publication

Not applicable.
The manuscript contains no individual person's data in any form (including individual details, images, or videos), so no consent to publish have been obtained from patients or their parent or legal guardian.

Competing interests

The authors declare that they have no competing interests.

Publisher's Note

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