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Understanding the role of the immune system in Adolescent Idiopathic Scoliosis: Immunometabolic CONnections to Scoliosis (ICONS) Study Protocol

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Abstract

Introduction: Adolescent Idiopathic Scoliosis (AIS) affects up to 3% of children around the world. There is limited knowledge to AIS etiopathogenesis, and this evidence is needed to develop new management strategies. Paraspinal muscle in AIS demonstrates evidence of differential fibrosis based on curve sidedness. Fibrosis is the hallmark of macrophage-driven inflammation and tissue remodeling, yet the mechanisms of fibrosis in paraspinal muscle in AIS are poorly understood.

Objectives: The primary objective of this study is to determine the influence of curve sidedness on paraspinal muscle inflammation.

Secondary objectives include determining the mechanisms of macrophage homing to muscle, and determining muscle-macrophage crosstalk in muscle fibrosis in AIS.

Methods and analysis: This is a cross-sectional study conducted in a tertiary pediatric center in Hamilton, Ontario, Canada. We will recruit boys and girls, 10-17 years of age who are having surgery to correct AIS. We will exclude children who have an active infection or are on immunosuppressive therapies within two weeks of surgery, smokers, and pregnant girls. Paraspinal muscle biopsies will be obtained at the start of surgery. Also, blood and urine samples will be collected from participants, who will fill questionnaires about their lifestyle. Anthropometric measures will also be collected including height, weight, waist and hip circumferences.

Ethics and dissemination: This study has received ethics authorization by the institutional review board. This work will be published in peer-reviewed journals, and will be presented in oral and poster formats at scientific meetings.

Discussion: This study will explore the mechanisms of paraspinal muscle inflammation, remodeling, and fibrosis in AIS. This will help identify pathways and molecules as potential therapeutic targets to treat and prevent AIS. It may also yield markers that predict scoliosis progression and response to treatment in these children.

Strengths:

- This is the first study to determine the mechanisms of inflammation and its effect of paraspinal muscle remodeling and fibrosis in AIS
- This study will also shed light on immune cell phenotype differences in paraspinal muscle on both sides of the scoliotic curve, and how this then drives immune-muscle crosstalk in AIS.

Limitations:

- Due to the cross-sectional nature of the study, it will not be possible to determine immune cell phenotype in muscle is a cause or effect of spinal curving.
- As samples are collected at the maximal points of convexity and concavity, potential changes in other parts of the paraspinal muscle will not be studied.

Introduction

Idiopathic scoliosis is a three-dimensional deformity of the spine that occurs in up to 3% of children globally(1). Adolescent Idiopathic Scoliosis (AIS) is the most common form(1, 2), and in 80% of cases occur in girls(3).

AIS has potential implications for health across the lifespan including pain, mobility problems, pulmonary hypertension, and psychological health issues. While some of these complications have been inconsistently reported, their occurrence places a heavy burden on the child, and some do not improve with treatment(4-6). It is a challenge to predict which child will develop AIS, and who is at risk of curve progression, which limits the development of precise therapies. The potential risk factors for progression of the scoliotic curve include curves with Cobb angle ≥ 30 degrees, pubertal growth spurt, premenarchal girls, a right thoracic curve in girls and left lumbar curve in boys(1, 7).

Current treatment options depend on the severity of the scoliotic curve. In patients who are still growing with a Cobb angle less than 25 degrees, observation in the mainstay of therapy. In cases with significant curvature (Cobb angle 25-40 degrees) or with worsening of the curve, patients may require bracing to help control progression, but this does not reduce the severity of the curve(8-13). In those who have completed growth or are still growing with significant curves (Cobb angle 45 degrees of higher), surgery is often undertaken to halt curve progression and induce partial correction(14-18). Bracing and surgical complications, although rare, are significant especially neurological complications in combined anterior and posterior surgical procedures(19, 20).

The mechanisms driving the initiation and propagation of the scoliotic curves are incompletely understood, and there is a paucity of models that can improve insights into the genesis of AIS. Animal models do not have the same spinal architecture of humans; assessing children when curve initiation timing is not known until the patient presentation for assessment is another challenge. Some models rely on chemical induction of scoliosis, so the faithful replication of etiopathogenesis of AIS in these models is not possible(21, 22). The prevalence of AIS, potential comorbidities, treatment-related complications, the lack of a unifying mechanism, and the dearth of models to provide mechanistic insights into AIS occurrence make this disease a significant challenge to solve. Understanding the etiopathogenesis of AIS continues to rely on studying humans as the best model of their disease.

Defining the mechanisms driving AIS will help the development of precise diagnostic, therapeutic and prevention strategies, which will likely improve outcomes.

Potential mechanisms driving AIS etiopathogenesis: It is clear that AIS is a polygenic disease with genetic, epigenetic, nervous system, hormonal/metabolic, biochemical, musculoskeletal, environmental and possible lifestyle factors contributing to its genesis(23, 24). Regardless of its cause, abnormalities of vertebral growth plates are a final destination for many proposed mechanisms that drive AIS. Still, there are many unanswered questions regarding the mechanisms involved in the development and progression of AIS.

Genetic Factors: AIS is more common in families with a history of the disease, but there is no definite mode of inheritance. The main hurdles to identifying genes of AIS pathogenesis is the heterogeneity of genotypic and phenotypic definitions of cases, and relatively small sample sizes included in genetic studies(25).

It is likely that many genes of minor effect contribute to the development of AIS. Twin studies have been inconsistent in showing increased concordance in monozygotic and dizygotic twins(26-30). This inconsistency suggests that other factors, including environmental and epigenetic elements, contribute to AIS with a given genetic background.

Gene linkage studies suggest that genetic factors influence the development of AIS, with loci so far on chromosomes 1,3,5,6,7,8,9,11,12,16,17,18 and 19 (25, 29, 31, 32).

Several candidate genes have been linked to AIS, but confirmation of these associations remain inconsistent, and requires larger replication studies(33-42). A two-phase model for the development of AIS has been proposed. Under this model, a set of genetic factors initiates the development of the curve, while another set determines curve progression. These sets of genes may overlap in their effects, and may be influenced by the environment(43).

Importantly, genome-wide association studies (GWAS) has implicated new genes, with one study creating a list of markers that are predictive of curve progression(44).

Recent GWAS studies reported the association of a gene expressed in the dorsal spinal cord, skeletal muscle and somatosensory neurons with AIS. Ladybird Homeobox 1 (LBX1) is involved in muscle precursor and neuronal cell development and migration(45-47).

A microduplication of the genomic region where LBX1 gene resides (10q24.31) was associated with scoliosis and myopathy(48). Further studies are needed to explore the mechanistic details of the role of LBX1 in AIS.

Epigenetics: Epigenetics refer to the phenomenon of production of different phenotypes due to changes in the expression of a gene rather than its sequence(49). Epigenetics has been

implicated as one of the mechanisms driving many non-communicable diseases due to exposure to an adverse intrauterine environment(50), but the evidence for epigenetics in AIS requires further study(23).

Environmental and Lifestyle Factors: Sporadic reports link diet (e.g. calcium intake), and low body-mass index (BMI) with the prevalence of AIS(51). Inadequate calcium intake during the peripubertal period might result in poor bone mineralization with accelerated bone growth(52, 53).

There is controversy as to whether girls with AIS have different body composition to girls with no AIS. Some studies have reported that body mass index (BMI) and fat mass are lower in AIS, but this has not been a consistent finding(54-56). The differences in BMI and adiposity that may affect spinal development and skeletal maturation, and their role in AIS is unknown. Also, lower physical activity has been studied as an association of AIS, and the speculation is that this is related to a proprioception defect(57). Later age of menarche has been associated with more risk of AIS, and the risk is reduced for those who live closer to the equator(58). Systematic studies of the role of environmental factors in AIS are needed.

The Nervous System: As patients with several neurological syndromes have scoliosis, there has been significant focus on defining neurological factors contributing to AIS(59-61). In addition, defects in central control and processing of information have been proposed to be associated with AIS, although their role in the development and propagation of AIS remains under investigation.

Anatomical abnormalities involving spine, midbrain, pons, medulla, vestibular and hindbrain regions have been reported in AIS(62-64). Differences in brain volume, internal capsule and corpus callosum were also reported(65-68). How these differences drive AIS is a mystery.

Neurophysiological mechanisms have also been reported in AIS, including abnormal proprioception, oculovestibular dysfunction, lateral gaze palsy, dynamic balance problems, postural imbalance and somatosensory disequilibrium(69-74). There have also been reports of enhanced electromyographic activity of the convex side of the spine(75).

These phenomena led to the hypothesis that a combination of abnormal spinal growth patterns and neuromuscular and tissue remodeling are implicated in AIS(76). Posture is determined by sensory input from visual, vestibular, and proprioception neural pathways, coupled with motor output. As a first step, postural disequilibrium due to defects in the neuromuscular system leads to the development of small spinal curves. With ongoing spinal growth, a second step involves biomechanical and neurological factors that drive the progression of the curve(76). With the establishment of scoliosis, secondary geometric and morphological changes emerge(77-79).

Hormones and Metabolic Dysfunction: Studies have produced mixed results on the association of several biomarkers, their receptors and gene variants with AIS. Some of the data have suggested that growth hormone (GH)(80, 81), calmodulin(82), melatonin(42, 82-87), and leptin[23] have a role in AIS.

Melatonin deficiency, triggered by pinealectomy, was shown to cause scoliosis(88-90), and melatonin levels were reported to be reduced in AIS patients(91). Both findings were not replicated in other studies that used pinealectomy or light stimulation to suppress melatonin in different models, including primates(87, 92, 93). In addition, patients with sleep disorders who have melatonin suppression are not at increased risk of scoliosis(94). One study suggested impairment in the melatonin signaling pathway in primary osteoblasts from AIS patients(95). It has been proposed that initial spinal imbalance is sustained by biochemical,

postural, and melatonin signaling pathways that contribute to the development of scoliosis(96). Further research is needed to define the potential role of melatonin in AIS.

Calmodulin is a regulator of contractile properties of muscle, and its increased levels may lead to altered paraspinal muscle activity and progression of the scoliotic curve. This hypothesis is supported by the fact that calmodulin is asymmetrically distributed across the paraspinal muscles of AIS patients(82).

Leptin has also been implicated in the genesis of AIS. The “leptin-hypothalamic-sympathetic nervous system theory” proposes that asymmetrical hypothalamic upregulation of leptin signaling leads to an asymmetrical activation of the sympathetic nervous system that may be associated with upregulation of GH/IGF-1 axis.

Since the sympathetic nervous system regulates the growing axial skeleton, the resulting asymmetrical vertebral growth plates may affect the progression of AIS, while the somatic nervous system may be involved in the initiation of the curve by the failure of mechanisms that maintain posture(97, 98).

Biomechanical Factors: The human spine has natural kyphotic curves at thoracic and sacral regions, and lordotic curves at cervical and lumbar regions. One consistent anomaly in AIS is the shape abnormality in the sagittal plane(99). A large study that evaluated children for scoliosis demonstrated that lordosis was always present prior to the development of thoracic AIS(100).One potential hypothesis that may explain the progression of the spinal curve in AIS is that AIS patients are taller that controls, and this is coupled with the fact that girls have more slender spines than boys(99). This makes the spine in girls more likely to buckle under force, and this is maximized during periods of rapid spinal growth including puberty. Increased compression force on the concave side may reduce spinal growth, while

reduced loading on the convex side may accelerate growth(99, 101). Another important study evaluated cadaveric idiopathic scoliosis demonstrated that the primary abnormality is lordosis with anterior vertebral wedging. This leads to biomechanical instability, and compensatory rotation and scoliosis(102).

Paraspinal muscle metabolism in AIS:

The fiber composition of paraspinal muscle in AIS has been studied and while the results are inconsistent, the prevailing view is that type I fibers are more prevalent on convex side of the curve. Type II fibers are increased or reduced on the concave side, but were increased in the convex side in one study(103-110).

Other abnormalities noted in muscle include increased intracellular calcium, reduced zinc(111), increased glucocorticoid receptors on the convex side(112), reduced RNA activity on the concave side and increased protein synthesis on the convex side(113). Muscle enzymes seem equal with minor exceptions(114).

Skeletal muscle-immune crosstalk: A novel paradigm in potential pathogenesis of AIS

While the literature hints at muscle phenotype as a secondary phenomenon for scoliosis, it is a difficult conclusion to establish.

One important consideration is that the diagnosis of AIS is taken to be the time of presentation. However, scoliosis would have evolved over an undefined period before the diagnosis, making longitudinal studies starting at inception a challenge. Therefore, muscle phenotype may drive or be driven by scoliosis.

It is challenging to obtain repeated muscle biopsies to determine muscle changes with scoliosis initiation and progression, as this is an invasive procedure and this approach ethical or feasible. All studies that have examined muscle pathology in AIS have done so in a cross-

sectional fashion due to these limitations(22, 115, 116). Animal models used to study this disease have relied on surgically or chemically-induced scoliosis, which may not faithfully recapitulate the events in patients, and requires specific expertise especially when using big animals (e.g. chicken).

Recent evidence suggests that while paraspinal muscle on both sides of the spinal curve has increased fibrosis, muscle on the concave side of the spine demonstrated more enhanced fibrosis and fatty involution compared to concave side(116). These findings were taken to suggest a myopathic process based on finding central core lesions in muscle. Importantly, fibrosis is the hallmark of immune cell-tissue interaction to remodel tissues and restore homeostasis(52, 117).

Innate immunity & fibrosis in AIS:

The innate immune system is the initial line of defense against environmental threats(118). Components of the innate immune system include cells (monocytes and neutrophils) and receptors including Nucleotide-Binding Oligomerization Domain (NOD) proteins and Toll-like receptors (TLRs)(119, 120). While acute activation in muscle injury results in a controlled remodeling response mediated by neutrophils and macrophages, chronic activation and low-grade inflammation can lead to dysregulation of tissue remodeling and fibrosis(117).

Circulating monocytes are attracted to different tissues in response to chemokines. Monocytes sense the muscle microenvironment and, guided by tissue cues, differentiate to macrophages(121). There are two main types of macrophages; inflammatory or ‘M1’ macrophages originate from bone marrow-derived monocytes that enter the injured tissue, and produce pro-inflammatory cytokines and are detected in muscle(122, 123). Resident or ‘M2’ macrophages are present in tissues under physiological conditions and help with tissue

homeostasis and remodeling(124). The imbalance between anti-inflammatory actions of M2 and inflammatory responses by M1 macrophages is a fundamental driver of the effects of inflammation on muscle phenotype. This M1 and M2 paradigm is a rather simplistic view of macrophage phenotype, and there are several intermediate phenotypes driven by tissue demands(125, 126). One possibility is that in AIS, muscle inflammation is an acute-on-chronic process, whereby repeated cycles of tissue injury related to the progression of the scoliotic curve may lead to concomitant inflammation and fibrosis. This is supported by paraspinal muscles demonstration of simultaneous atrophy and hypertrophy, indicating that there is the ongoing activity to remodel muscle(116). The tissue injury may be the primary initiating event or the result of spinal curvature.

Recent evidence has strengthened the evidence for the role of macrophages in muscle fibrosis in a chronic muscle disease, Duchenne Muscular Dystrophy(127). Acute muscle injury is characterized by three important responses: 1) expansion of satellite cells (muscle stem cells), 2) infiltration of inflammatory 'M1' macrophages, and 3) expansion of resident mesenchymal cells (fibro/adipogenic precursor cells; FAPs). The latter two mechanisms regulate myogenesis, whereby the expansion of FAPs in acute injury is followed by an apoptotic response to regulate FAPs mass; Tumor Necrosis Factor Alpha (TNFα), a prototypical inflammatory cytokine, is secreted by infiltrating inflammatory macrophages and drives this response. This allows limited FAPs expansion and regulates tissue remodeling to restore normal tissue function(127).

On the other hand, chronic or repeated muscle injury (which may be a primary or secondary event in AIS) triggers a tissue repair response. This response is characterized by expansion of pro-generation (M2) macrophages, activation of FAPs, and the production by M2

macrophages of Transforming Growth Factor beta1 (TGFβ1), which inhibits TNFα and allows FAP expansion. This is associated with enhanced fibrosis and extracellular matrix deposition in muscle(127). Therefore, the shift from pro-myogenic to pro-fibrotic muscle response is driven by macrophage population differences. Whether this translates to AIS is unclear.

In addition, other immune cells including Neutrophils and T-Lymphocytes are likely to play a role in muscle Immunometabolism, but limited data exist as to their role in AIS.

Immune cells and the chemokine(s) that attract them to paraspinal muscle in AIS have not been studied. However, they may contribute to the initiation or propagation (or both) of the spinal curve in AIS by influencing muscle remodeling. In addition, muscle-bone-cartilage-disc-immune crosstalk at the interphase of these tissues that reside nearby is critically important to tissue metabolism and health(128) but has not been studied in AIS. Understanding the role of the immune system in muscle inflammation, metabolism, and fibrosis in AIS is the objective of the proposed study.

The role of skeletal muscle inflammation and fibrosis in the causation of AIS is not completely understood. It is imperative to delineate the role of immune cells in muscle in AIS, as this may help define targeted therapies to muscle immune cells, define biomarkers of scoliosis initiation, progression, and response to therapy.

The Immune-metabolic CONnections to Scoliosis (ICONS) study was designed to investigate paraspinal muscle-immune crosstalk and its role in AIS.

Research question:

In adolescents with AIS, does paraspinal muscle on the concave side of the scoliotic curve, compared to paraspinal muscle on the convex side, have enhanced anti-inflammatory

macrophage infiltration? If so, is this associated with fibro/adipogenic progenitor expansion and fibrosis?

Objectives:

Primary: To quantify macrophages and muscle inflammation in paraspinal muscle from concave and convex sides of the scoliotic curve

Secondary:

1. Profile chemokines in paraspinal muscle from concave and convex sides of the scoliotic curve.

2. Assess the role of fibro/adipogenic progenitor (FAP) in muscle fibrosis in AIS.

Hypothesis: In adolescents with AIS, enhanced anti-inflammatory macrophage infiltration of paraspinal muscle on the concave side, compared to the convex side of the curve, drives fibro/adipogenic progenitor expansion and differential fibrosis.

Methods & study procedures

Study design

This is a cross-sectional study. The participants will be recruited from Pediatric Orthopedic Clinic at McMaster Children's Hospital, a tertiary pediatric care center in Hamilton, Ontario, Canada. The study procedures are described in Figure 1.

Cohort characteristics

Inclusion criteria: We will include boys and girls, 10-17 years of age with a diagnosis of AIS confirmed on clinical and radiological grounds, and who have been informed by their team that they require and have agreed to undergo spinal surgery. We will include lean [BMI centile below 85th] and overweight/obese [BMI centile ≥85th] who are free from

infection for 15 days before sample collection.

Exclusion criteria: We will exclude patients with scoliosis related to other causes including congenital scoliosis, neuromuscular disorders, metabolic disorders, skeletal dysplasia, and syndromes. We will also exclude those on medications used within 15 days prior to sample collection (including high dose steroids, immunosuppressive therapy, and anti-thrombotic medications). We will also exclude those with active bacterial, viral or fungal infections, and chronic inflammatory diseases including autoimmune disease [Systemic Lupus Erythematosis, Juvenile Idiopathic Arthritis, Dermatomyositis]. We will also exclude smokers, pregnant girls and those who are unable or unwilling to provide signed consents.

Recruitment & consents:

The ICONS study will recruit patients who have been deemed eligible for scoliosis surgery. The healthcare provider will ask permission from potential participants to be approached by the study team. If patients and parents agree, the study team will meet the participants on the day of their preoperative evaluation to introduce the study and answer questions.

If the family and participant agree to participate, consent forms will be signed. The consent forms include those for parents and assent forms for children between 10-15 years of age. For those 16 years or older, the participants will sign their consents. Separate consent forms for genetic (DNA) testing are completed. The participants are assigned unique identifying numbers to protect confidentiality and data are anonymized shortly after collection.

Questionnaires:

After consent procedures are completed, study questionnaires are provided to the participants and their families.

The study will collect sociodemographic and clinical data including age, gender, grade in school, parental education, religion, ethnicity, history of medical or surgical problems, birth history, family income, social history, and family history of medical problems.

Regarding dietary information, the intake of different food items will be documented using a dietary questionnaire modified from the Adolescent Food Frequency Questionnaire(129, 130). Reported physical activity will be measured using the HAES questionnaire(131). Sleep will be measured using the Chevrin sleep questionnaire(132). We will also enquire about mental health by asking participants or parents to fill a questionnaire reporting mental health problems(133). Neighborhood walkability will be determined using the NEWS questionnaire(134).

Anthropometric & clinical measurements:

The participant will have their height measured closest to 0.1 cm using a stadiometer, weight to closest 0.1 kg using weighing scale, and Body Mass Index (BMI) in kg/m^2 will be calculated from height and weight and BMI centile determined from CDC growth charts. The waist circumference and hip circumference will be measured using a spring-loaded measuring tape closest to 0.1 cm. Puberty will be assessed using drawings describing Tanner staging for girls eight years or older, and external genitalia for boys nine years and older(135).

Sitting systolic and diastolic blood pressure (BP) will be measured twice using automated BP monitor, and pulse rate is measured using same device. Body fat percentage is measured

using Tanita body fat monitor for children (Tanita Corporation, Illinois, USA), and grip strength will be tested using a Dynamometer.

Biological samples:

All samples will be obtained on the day of surgery after an overnight fast of 8-10 hours.

Blood

Blood samples are obtained after patients are anesthetized and central lines are in place to permit blood sampling. We will be collecting serum and plasma samples as well as buffy coat for DNA studies. PAX RNA whole blood samples are also collected for RNA studies. Samples will be aliquoted to cryovials, and stored at -80 °C until further use.

Urine

Urine samples will be obtained using 90 ml urine containers after urinary catheterization for the surgical procedure. Urine is aliquoted to cryovials and stored at -80 °C until further use.

Tissue sampling and processing:

Paraspinal muscle (erector spinae) samples will be collected shortly after starting the surgical procedure for correction of scoliosis. The points of sampling include samples from the apex (maximal area of convexity) of the spinal curve, matched with a biopsy from the area of maximal concavity on the opposite side of the spinal curve.

Two important procedures are applied during sample collection that will ensure fidelity of immune cell phenotype. First, we will collect the samples before adrenaline injections, used to control bleeding during surgery, as it is known that adrenergic stimuli may change macrophage responses. We will also avoid cauterized areas of tissue sampling.

The muscle samples will be cleaned from blood by drying with gauze, and connective tissue is separated from samples. Tissue samples are then divided into five pieces, and four pieces will be snap frozen immediately in liquid nitrogen and transferred to -80 °C freezer for storage. One piece will be formalin-fixed and paraffin-embedded until further processing.

Experimental work details:

Primary objective: Determine the influence of curve sidedness on paraspinal muscle inflammation

Muscle macrophage content will be profiled using CD68 Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR), western blot and Immunohistochemistry (IHC)(136, 137).

For qRT-PCR, muscle tissue (25-50 mg) will be chipped and powdered, and then added to reagent. The tissue will be homogenized, and RNA isolation will take place using RNAeasy minikit (Qiagen).

One microgram of RNA will be used to generate cDNA utilizing SuperScript III reverse transcriptase kit (Invitrogen, Carlsbad, CA). Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) will be completed using TaqMan assay primer-probe mixes (Applied Biosystems) as we previously described(136, 138). Western blot and Immunohistochemistry will be performed as described previously by our group(136-138).

The full characterization of macrophage phenotype will be determined by testing markers of inflammatory macrophages including Cluster of Differentiation-11c (CD11c), Arginase II, and C-C chemokine receptor 2 (CCR2). Anti-inflammatory macrophages will be detected by using Cluster of Differentiation-206 (CD206), TGFβ1, and Cluster of Differentiation 301 (CD301) using qRT-PCR, western blot and IHC co-staining methods(139). We will use Nikon eclipse 90i microscope for imaging, and analyze images to determine macrophage content

using NIS Element 64 bit 3.22.11 Software (Nikon Inc., Melville, NY)(136, 137, 140).

To profile muscle inflammation, we will perform qRT-PCR using TaqMan assay primer-probe mixes (Applied Biosystems) on cDNA synthesized from RNA from muscle.
We will test the gene expression of pro-inflammatory TNFα, Interleukin-1β (IL-1β), Interleukin-6 (IL-6) and Interferon gamma (INFγ). Anti-inflammatory cytokines measured Include Interleukin-10 (IL-10) and Interleukin-1 receptor antagonist (IL-1Ra).
We will also measure cytokines in lysates from muscle (TNFα, IL-1β, IL-6, INFγ, IL-10, IL-1Ra) using Bio-Plex assays (Bio-Rad) as we previously reported(137).
Activation of inflammatory pathways responsible for cytokine & chemokine production will be tested using western blot including c-Jun-N-Terminal kinase (JNK), Extracellular signal-regulated kinase (ERK), p38 Mitogen Activated Protein Kinase (p38MAPK), and NFκB pathways (Cell signaling)(138).
Total and phosphorylated (activated) versions of the molecules will be quantified, and reported as a ratio of phosphorylated/total protein. GAPDH will be used as a loading control(138).

Secondary objectives:

1-Profile chemokines in paraspinal muscle from concave and convex sides of the scoliotic curve.

As chemokines drive macrophage migration to muscle, there is a need to determine the chemokines driving macrophage infiltration to muscle in AIS. We will assess the gene expression of CCL2, CCL3, CCL5, Macrophage Colony Stimulation Factor (MCSF), Granulocyte-Monocyte Colony Stimulation Factor (GM-CSF), and Chemokine (C-C) Ligand

20 (CCL20), which accounts for most muscle chemokinetic activity.

Chemokine protein content (CCL2, CCL3, CCL5, MCSF, GM-CSF, CCL20) will be assessed using Bio-Plex assays (Bio-Rad) as we previously reported(137).

2-Assess the role of fibro/adipogenic progenitor (FAP) in muscle fibrosis in AIS.

The experimental design of this part is described in Figure 2. We will generate macrophages and evaluate inflammatory (M1) and anti-inflammatory (M2) effects on FAPs. We will isolate FAPs from paraspinal muscle using $CD15^{+}PDGFR\alpha^{+}CD56^{-}$ markers after Collagenase digestion by flow cytometry(141).

To generate macrophages, we will differentiate THP-1 human monocyte cell line to macrophages (24 h incubation with 150 nM phorbol 12-myristate 13-acetate (PMA), followed by 24 h incubation in RPMI medium). Macrophages will then be polarized to M1 macrophages (20 ng/ml IFNγ+10 pg/ml LPS). Polarization to M2 will be done by incubating cells in 20 ng/ml IL-4+ 20 ng/ml IL-13)(142). We will collect the medium M1 and M2 cells are growing in. This conditioned medium from M1 (CMM1) and M2 (CMM2) will be used in this set of experiments.

To determine if inflammatory (M1) macrophages produce factors that reduce FAP proliferation and differentiation, we will treat FAPs with CMM1 for 24h. We will then measure FAP proliferation with BrdU incorporation assay, differentiation to adipocytes using oil red O staining, and differentiation to fibroblasts using gene expression and western blot of ER-TR7, FSP-1, and alpha smooth muscle actin. We will also test FAPs for apoptosis by performing TUNEL assay using TiterTACS® in situ kits (R&D Systems).

We will then measure CMM1 cytokines including TNFα using ELISA, and use TNFα neutralizer Adalimumab, and repeat FAP treatment(143). In addition, we will treat FAPs

with TNFα receptor antagonist (R-7050; Santa Cruz) (144) and repeat the experiments. To determine if anti-inflammatory (M2) macrophages produce factors that enhance FAP proliferation and differentiation, we will treat FAPs with CMM2 for 24h. We will then measure FAP proliferation with BrdU incorporation assay, differentiation to adipocytes using oil red O staining, differentiation to fibroblasts using gene expression and western blot of ER-TR7, FSP-1, and alpha smooth muscle actin, and apoptosis by performing TUNEL assay. We will then measure CMM2 cytokines including TGFβ, and then use TGFβ neutralizing antibodies and test FAP proliferation and differentiation. We will also treat FAPs with TGFβ receptor antagonist (LY2157299; Eli Lilly)(145), and repeat the FAP treatment with CMM2.

Statistical analysis:

Outcome variables will be summarized using descriptive summary measures expressed as mean (standard deviation) or median (minimum-maximum) for continuous variables, and number (%) for categorical variables. Data will be checked for normality using Kilmogorov-Smirnoff test, and log transformed if not Normally distributed. We will test differences in socio-demographic and clinical data between groups using Chi-square tests for categorical variables and T-test or Kruskall Wallis tests for continuous variables depending on the distribution. Statistical analysis of qRT-PCR experiments will be done using ΔΔCT(136). For ELISA, standard curves will be

generated and cytokines normalized to total protein content of the sample and reported as pg μL^{-1} μg^{-1} protein(137). For western blots, we will measure the total and phosphorylated protein and generate a ratio of total/phosphoprotein for comparisons (22).

For IHC, Macrophages will be counted manually at x400 magnification, and the number of macrophages is normalized to the section area. The researcher counting the cells will be blinded to the experimental group allocations as we previously reported (17).

For human samples, all analyses will be performed using regression analyses to pool and compare the groups adjusting for age, sex, BMI, ethnicity, and fitness. For in-vitro and murine studies, clinical data will be analyzed using T-test.

The results will be reported as estimates of the difference, corresponding 95% confidence intervals and associated p-values. Alpha is set at 0.05, adjusted using Bonferroni approach for multiple analyses. All analyses will be performed using SAS 9.2 (Cary, NC).

Sample Size: For sample size calculations for the full study, as macrophage content in paraspinal muscle has not been done in AIS before, power calculation is difficult. We aim to recruit 120 patients based on sample size calculations according to Norman & Streiner(146), whereby 10 patients are needed per marker studied. We plan to approach 200 patients, and assuming 80% recruitment rate, and 20% attrition, with one patient being evaluated weekly for surgery and 40 scoliosis surgeries per year at our institution, we plan to complete patient recruitment over 60 months for the full study.

Discussion:

Previous research has demonstrated the presence of fibrosis in paraspinal muscle in AIS(116). The immune system is one of the major players in muscle tissue remodeling via inflammation, yet this has not been studied in AIS.

This work will focus on understanding the mechanisms driving immune-muscle crosstalk in AIS, and the contribution of FAPs to muscle fibrosis. This work will aid the development of anti-inflammatory and anti-fibrotic therapies to help retain muscle strength, reduce pain, and delay or prevent the development of the scoliotic curve. This study may also reveal muscle-specific biomarkers that predict initiation and progression of the scoliotic curve, response to therapy, or the development of complications.

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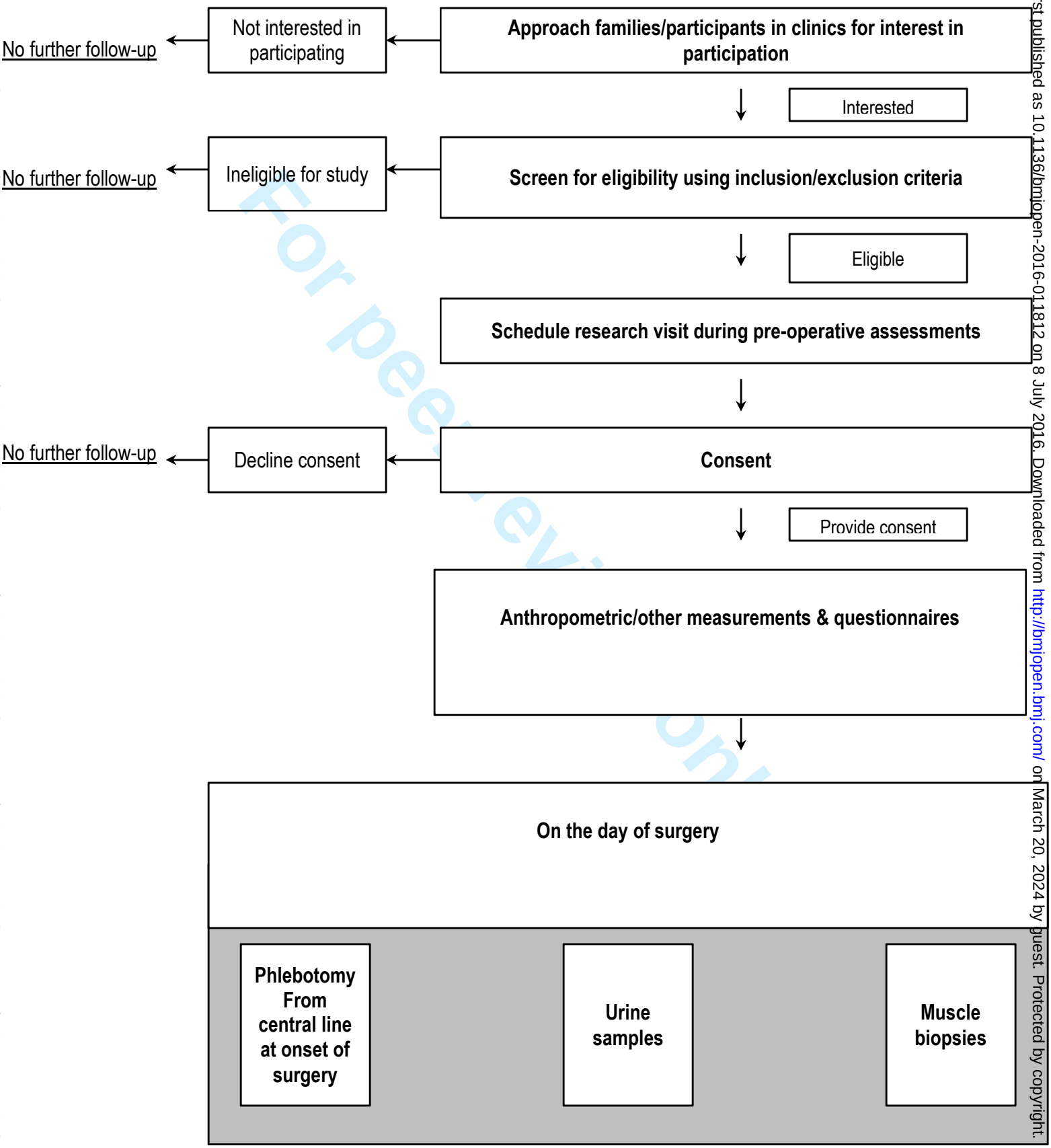

Figure 1: ICONS study flow diagram

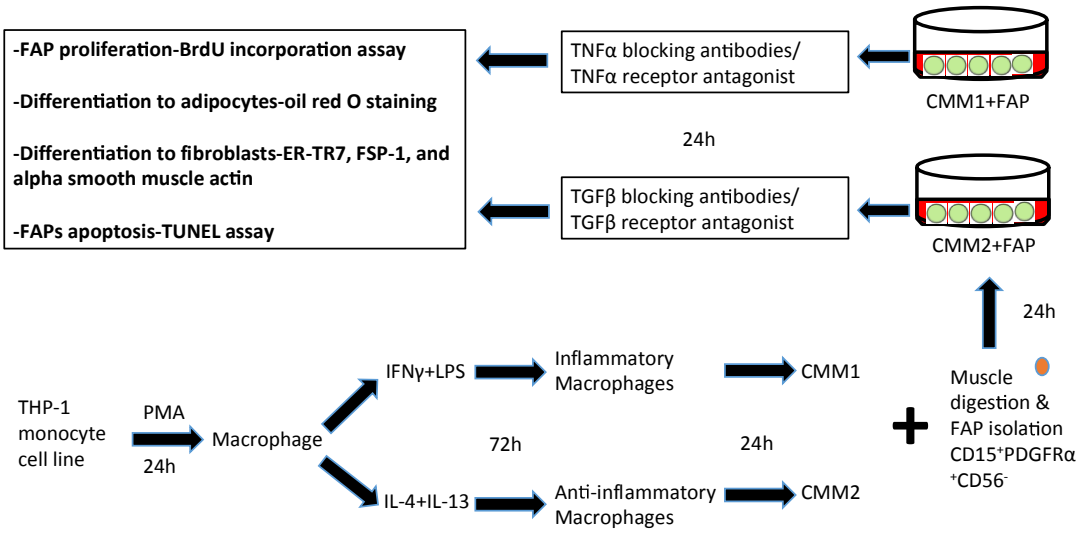

Figure 2: Testing macrophage-muscle crosstalk in AIS. THP-1 monocyte cell line is treated with 150 nM phorbol 12-myristate 13-acetate (PMA) for 24 hours to generate macrophages. Macrophages are treated with 20 ng/ml IFNγ+10 pg/ml LPS to generate inflammatory macrophages, and 20 ng/ml IL-4+ 20 ng/ml IL-13 to generate anti-inflammatory macrophages. The medium the cells are growing in is called conditioned medium (CM) for inflammatory (CMM1) and anti-inflammatory (CMM2) macrophages. To generate fibro/adipogenic progenitors (FAPs), muscle will be digested with Collagenase, and FAPs will be plated into 12 well plates. We will then add CMM1 and CMM2 to FAPs, and then test FAP proliferation, differentiation to adipocytes and fibroblasts, and apoptosis.

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Understanding the role of the immune system in Adolescent Idiopathic Scoliosis: Immunometabolic CONnections to Scoliosis (ICONS) Study Protocol

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Abstract

Introduction: Adolescent Idiopathic Scoliosis (AIS) affects up to 3% of children around the world. There is limited knowledge to AIS etiopathogenesis, and this evidence is needed to develop new management strategies. Paraspinal muscle in AIS demonstrates evidence of differential fibrosis based on curve sidedness. Fibrosis is the hallmark of macrophage-driven inflammation and tissue remodeling, yet the mechanisms of fibrosis in paraspinal muscle in AIS are poorly understood.

Objectives: The primary objective of this study is to determine the influence of curve sidedness on paraspinal muscle inflammation.

Secondary objectives include determining the mechanisms of macrophage homing to muscle, and determining muscle-macrophage crosstalk in muscle fibrosis in AIS.

Methods and analysis: This is a cross-sectional study conducted in a tertiary pediatric center in Hamilton, Ontario, Canada. We will recruit boys and girls, 10-17 years of age who are having surgery to correct AIS. We will exclude children who have an active infection or are on immunosuppressive therapies within two weeks of surgery, smokers, and pregnant girls. Paraspinal muscle biopsies will be obtained at the start of surgery. Also, blood and urine samples will be collected from participants, who will fill questionnaires about their lifestyle. Anthropometric measures will also be collected including height, weight, waist and hip circumferences.

Ethics and dissemination: This study has received ethics authorization by the institutional review board. This work will be published in peer-reviewed journals, and will be presented in oral and poster formats at scientific meetings.

Discussion: This study will explore the mechanisms of paraspinal muscle inflammation, remodeling, and fibrosis in AIS. This will help identify pathways and molecules as potential therapeutic targets to treat and prevent AIS. It may also yield markers that predict scoliosis progression and response to treatment in these children.

Strengths:

- This is the first study to determine the mechanisms of inflammation and its effect of paraspinal muscle remodeling and fibrosis in AIS
- This study will also shed light on immune cell phenotype differences in paraspinal muscle on both sides of the scoliotic curve, and how this then drives immune-muscle crosstalk in AIS.

Limitations:

- Due to the cross-sectional nature of the study, it will not be possible to determine immune cell phenotype in muscle is a cause or effect of spinal curving.
- As samples are collected at the maximal points of convexity and concavity, potential changes in other parts of the paraspinal muscle will not be studied.

Introduction

Idiopathic scoliosis is a three-dimensional deformity of the spine that occurs in up to 3% of children globally(1). Adolescent Idiopathic Scoliosis (AIS) is the most common form(1, 2), and in 80% of cases occur in girls(3).

AIS has potential implications for health across the lifespan including pain, mobility problems, pulmonary hypertension, and psychological health issues. While some of these complications have been inconsistently reported, their occurrence places a heavy burden on the child, and some do not improve with treatment(4-6). It is a challenge to predict which child will develop AIS, and who is at risk of curve progression, which limits the development of precise therapies. The potential risk factors for progression of the scoliotic curve include curves with Cobb angle ≥ 30 degrees, pubertal growth spurt, premenarchal girls, a right thoracic curve in girls and left lumbar curve in boys(1, 7).

Current treatment options depend on the severity of the scoliotic curve. In patients who are still growing with a Cobb angle less than 25 degrees, observation in the mainstay of therapy. In cases with significant curvature (Cobb angle 25-40 degrees) or with worsening of the curve, patients may require bracing to help control progression, but this does not reduce the severity of the curve(8-13). In those who have completed growth or are still growing with significant curves (Cobb angle 45 degrees of higher), surgery is often undertaken to halt curve progression and induce partial correction(14-18). Bracing and surgical complications, although rare, are significant especially neurological complications in combined anterior and posterior surgical procedures(19, 20).

The mechanisms driving the initiation and propagation of the scoliotic curves are incompletely understood, and there is a paucity of models that can improve insights into the genesis of AIS. Animal models do not have the same spinal architecture of humans; assessing children when curve initiation timing is not known until the patient presentation for assessment is another challenge. Some models rely on chemical induction of scoliosis, so the faithful replication of etiopathogenesis of AIS in these models is not possible(21, 22). The prevalence of AIS, potential comorbidities, treatment-related complications, the lack of a unifying mechanism, and the dearth of models to provide mechanistic insights into AIS occurrence make this disease a significant challenge to solve. Understanding the etiopathogenesis of AIS continues to rely on studying humans as the best model of their disease.

Defining the mechanisms driving AIS will help the development of precise diagnostic, therapeutic and prevention strategies, which will likely improve outcomes.

Potential mechanisms driving AIS etiopathogenesis: It is clear that AIS is a polygenic disease with genetic, epigenetic, nervous system, hormonal/metabolic, biochemical, musculoskeletal, environmental and possible lifestyle factors contributing to its genesis(23, 24). Regardless of its cause, abnormalities of vertebral growth plates are a final destination for many proposed mechanisms that drive AIS. Still, there are many unanswered questions regarding the mechanisms involved in the development and progression of AIS.

Genetic Factors: AIS is more common in families with a history of the disease, but there is no definite mode of inheritance. The main hurdles to identifying genes of AIS pathogenesis is the heterogeneity of genotypic and phenotypic definitions of cases, and relatively small sample sizes included in genetic studies(25).

It is likely that many genes of minor effect contribute to the development of AIS. Twin studies have been inconsistent in showing increased concordance in monozygotic and dizygotic twins(26-30). This inconsistency suggests that other factors, including environmental and epigenetic elements, contribute to AIS with a given genetic background.

Gene linkage studies suggest that genetic factors influence the development of AIS, with loci so far on chromosomes 1,3,5,6,7,8,9,11,12,16,17,18 and 19 (25, 29, 31, 32).

Several candidate genes have been linked to AIS, but confirmation of these associations remain inconsistent, and requires larger replication studies(33-42). A two-phase model for the development of AIS has been proposed. Under this model, a set of genetic factors initiates the development of the curve, while another set determines curve progression. These sets of genes may overlap in their effects, and may be influenced by the environment(43).

Importantly, genome-wide association studies (GWAS) has implicated new genes, with one study creating a list of markers that are predictive of curve progression(44).

Recent GWAS studies reported the association of a gene expressed in the dorsal spinal cord, skeletal muscle and somatosensory neurons with AIS. Ladybird Homeobox 1 (LBX1) is involved in muscle precursor and neuronal cell development and migration(45-47).

A microduplication of the genomic region where LBX1 gene resides (10q24.31) was associated with scoliosis and myopathy(48). Further studies are needed to explore the mechanistic details of the role of LBX1 in AIS.

Epigenetics: Epigenetics refer to the phenomenon of production of different phenotypes due to changes in the expression of a gene rather than its sequence(49). Epigenetics has been

implicated as one of the mechanisms driving many non-communicable diseases due to exposure to an adverse intrauterine environment(50), but the evidence for epigenetics in AIS requires further study(23).

Environmental and Lifestyle Factors: Sporadic reports link diet (e.g. calcium intake), and low body-mass index (BMI) with the prevalence of AIS(51). Inadequate calcium intake during the peripubertal period might result in poor bone mineralization with accelerated bone growth(52, 53).

There is controversy as to whether girls with AIS have different body composition to girls with no AIS. Some studies have reported that body mass index (BMI) and fat mass are lower in AIS, but this has not been a consistent finding(54-56). The differences in BMI and adiposity that may affect spinal development and skeletal maturation, and their role in AIS is unknown. Also, lower physical activity has been studied as an association of AIS, and the speculation is that this is related to a proprioception defect(57). Later age of menarche has been associated with more risk of AIS, and the risk is reduced for those who live closer to the equator(58). Systematic studies of the role of environmental factors in AIS are needed.

The Nervous System: As patients with several neurological syndromes have scoliosis, there has been significant focus on defining neurological factors contributing to AIS(59-61). In addition, defects in central control and processing of information have been proposed to be associated with AIS, although their role in the development and propagation of AIS remains under investigation.

Anatomical abnormalities involving spine, midbrain, pons, medulla, vestibular and hindbrain regions have been reported in AIS(62-64). Differences in brain volume, internal capsule and corpus callosum were also reported(65-68). How these differences drive AIS is a mystery.

Neurophysiological mechanisms have also been reported in AIS, including abnormal proprioception, oculovestibular dysfunction, lateral gaze palsy, dynamic balance problems, postural imbalance and somatosensory disequilibrium(69-74). There have also been reports of enhanced electromyographic activity of the convex side of the spine(75).

These phenomena led to the hypothesis that a combination of abnormal spinal growth patterns and neuromuscular and tissue remodeling are implicated in AIS(76). Posture is determined by sensory input from visual, vestibular, and proprioception neural pathways, coupled with motor output. As a first step, postural disequilibrium due to defects in the neuromuscular system leads to the development of small spinal curves. With ongoing spinal growth, a second step involves biomechanical and neurological factors that drive the progression of the curve(76). With the establishment of scoliosis, secondary geometric and morphological changes emerge(77-79).

Hormones and Metabolic Dysfunction: Studies have produced mixed results on the association of several biomarkers, their receptors and gene variants with AIS. Some of the data have suggested that growth hormone (GH)(80, 81), calmodulin(82), melatonin(42, 82-87), and leptin[23] have a role in AIS.

Melatonin deficiency, triggered by pinealectomy, was shown to cause scoliosis(88-90), and melatonin levels were reported to be reduced in AIS patients(91). Both findings were not replicated in other studies that used pinealectomy or light stimulation to suppress melatonin in different models, including primates(87, 92, 93). In addition, patients with sleep disorders who have melatonin suppression are not at increased risk of scoliosis(94). One study suggested impairment in the melatonin signaling pathway in primary osteoblasts from AIS patients(95). It has been proposed that initial spinal imbalance is sustained by biochemical,

postural, and melatonin signaling pathways that contribute to the development of scoliosis(96). Further research is needed to define the potential role of melatonin in AIS.

Calmodulin is a regulator of contractile properties of muscle, and its increased levels may lead to altered paraspinal muscle activity and progression of the scoliotic curve. This hypothesis is supported by the fact that calmodulin is asymmetrically distributed across the paraspinal muscles of AIS patients(82).

Leptin has also been implicated in the genesis of AIS. The “leptin-hypothalamic-sympathetic nervous system theory” proposes that asymmetrical hypothalamic upregulation of leptin signaling leads to an asymmetrical activation of the sympathetic nervous system that may be associated with upregulation of GH/IGF-1 axis.

Since the sympathetic nervous system regulates the growing axial skeleton, the resulting asymmetrical vertebral growth plates may affect the progression of AIS, while the somatic nervous system may be involved in the initiation of the curve by the failure of mechanisms that maintain posture(97, 98).

Biomechanical Factors: The human spine has natural kyphotic curves at thoracic and sacral regions, and lordotic curves at cervical and lumbar regions. One consistent anomaly in AIS is the shape abnormality in the sagittal plane(99). A large study that evaluated children for scoliosis demonstrated that lordosis was always present prior to the development of thoracic AIS(100).One potential hypothesis that may explain the progression of the spinal curve in AIS is that AIS patients are taller that controls, and this is coupled with the fact that girls have more slender spines than boys(99). This makes the spine in girls more likely to buckle under force, and this is maximized during periods of rapid spinal growth including puberty. Increased compression force on the concave side may reduce spinal growth, while

reduced loading on the convex side may accelerate growth(99, 101). Another important study evaluated cadaveric idiopathic scoliosis demonstrated that the primary abnormality is lordosis with anterior vertebral wedging. This leads to biomechanical instability, and compensatory rotation and scoliosis(102).

Paraspinal muscle metabolism in AIS:

The fiber composition of paraspinal muscle in AIS has been studied and while the results are inconsistent, the prevailing view is that type I fibers are more prevalent on convex side of the curve. Type II fibers are increased or reduced on the concave side, but were increased in the convex side in one study(103-110).

Other abnormalities noted in muscle include increased intracellular calcium, reduced zinc(111), increased glucocorticoid receptors on the convex side(112), reduced RNA activity on the concave side and increased protein synthesis on the convex side(113). Muscle enzymes seem equal with minor exceptions(114).

Skeletal muscle-immune crosstalk: A novel paradigm in potential pathogenesis of AIS

While the literature hints at muscle phenotype as a secondary phenomenon for scoliosis, it is a difficult conclusion to establish.

One important consideration is that the diagnosis of AIS is taken to be the time of presentation. However, scoliosis would have evolved over an undefined period before the diagnosis, making longitudinal studies starting at inception a challenge. Therefore, muscle phenotype may drive or be driven by scoliosis.

It is challenging to obtain repeated muscle biopsies to determine muscle changes with scoliosis initiation and progression, as this is an invasive procedure and this approach ethical or feasible. All studies that have examined muscle pathology in AIS have done so in a cross-

sectional fashion due to these limitations(22, 115, 116). Animal models used to study this disease have relied on surgically or chemically-induced scoliosis, which may not faithfully recapitulate the events in patients, and requires specific expertise especially when using big animals (e.g. chicken).

Recent evidence suggests that while paraspinal muscle on both sides of the spinal curve has increased fibrosis, muscle on the concave side of the spine demonstrated more enhanced fibrosis and fatty involution compared to concave side(116). These findings were taken to suggest a myopathic process based on finding central core lesions in muscle. Importantly, fibrosis is the hallmark of immune cell-tissue interaction to remodel tissues and restore homeostasis(52, 117).

Innate immunity & fibrosis in AIS:

The innate immune system is the initial line of defense against environmental threats(118). Components of the innate immune system include cells (monocytes and neutrophils) and receptors including Nucleotide-Binding Oligomerization Domain (NOD) proteins and Toll-like receptors (TLRs)(119, 120). While acute activation in muscle injury results in a controlled remodeling response mediated by neutrophils and macrophages, chronic activation and low-grade inflammation can lead to dysregulation of tissue remodeling and fibrosis(117).

Circulating monocytes are attracted to different tissues in response to chemokines. Monocytes sense the muscle microenvironment and, guided by tissue cues, differentiate to macrophages(121). There are two main types of macrophages; inflammatory or ‘M1’ macrophages originate from bone marrow-derived monocytes that enter the injured tissue, and produce pro-inflammatory cytokines and are detected in muscle(122, 123). Resident or ‘M2’ macrophages are present in tissues under physiological conditions and help with tissue

homeostasis and remodeling(124). The imbalance between anti-inflammatory actions of M2 and inflammatory responses by M1 macrophages is a fundamental driver of the effects of inflammation on muscle phenotype. This M1 and M2 paradigm is a rather simplistic view of macrophage phenotype, and there are several intermediate phenotypes driven by tissue demands(125, 126). One possibility is that in AIS, muscle inflammation is an acute-on-chronic process, whereby repeated cycles of tissue injury related to the progression of the scoliotic curve may lead to concomitant inflammation and fibrosis. This is supported by paraspinal muscles demonstration of simultaneous atrophy and hypertrophy, indicating that there is the ongoing activity to remodel muscle(116). The tissue injury may be the primary initiating event or the result of spinal curvature.

Recent evidence has strengthened the evidence for the role of macrophages in muscle fibrosis in a chronic muscle disease, Duchenne Muscular Dystrophy(127). Acute muscle injury is characterized by three important responses: 1) expansion of satellite cells (muscle stem cells), 2) infiltration of inflammatory 'M1' macrophages, and 3) expansion of resident mesenchymal cells (fibro/adipogenic precursor cells; FAPs). The latter two mechanisms regulate myogenesis, whereby the expansion of FAPs in acute injury is followed by an apoptotic response to regulate FAPs mass; Tumor Necrosis Factor Alpha (TNFα), a prototypical inflammatory cytokine, is secreted by infiltrating inflammatory macrophages and drives this response. This allows limited FAPs expansion and regulates tissue remodeling to restore normal tissue function(127).

On the other hand, chronic or repeated muscle injury (which may be a primary or secondary event in AIS) triggers a tissue repair response. This response is characterized by expansion of pro-generation (M2) macrophages, activation of FAPs, and the production by M2

macrophages of Transforming Growth Factor beta1 (TGFβ1), which inhibits TNFα and allows FAP expansion. This is associated with enhanced fibrosis and extracellular matrix deposition in muscle(127). Therefore, the shift from pro-myogenic to pro-fibrotic muscle response is driven by macrophage population differences. Whether this translates to AIS is unclear.

In addition, other immune cells including Neutrophils and T-Lymphocytes are likely to play a role in muscle Immunometabolism, but limited data exist as to their role in AIS.

Immune cells and the chemokine(s) that attract them to paraspinal muscle in AIS have not been studied. However, they may contribute to the initiation or propagation (or both) of the spinal curve in AIS by influencing muscle remodeling. In addition, muscle-bone-cartilage-disc-immune crosstalk at the interphase of these tissues that reside nearby is critically important to tissue metabolism and health(128) but has not been studied in AIS. Understanding the role of the immune system in muscle inflammation, metabolism, and fibrosis in AIS is the objective of the proposed study.

The role of skeletal muscle inflammation and fibrosis in the causation of AIS is not completely understood. It is imperative to delineate the role of immune cells in muscle in AIS, as this may help define targeted therapies to muscle immune cells, define biomarkers of scoliosis initiation, progression, and response to therapy.

The Immune-metabolic CONnections to Scoliosis (ICONS) study was designed to investigate paraspinal muscle-immune crosstalk and its role in AIS.

Research question:

In adolescents with AIS, does paraspinal muscle on the concave side of the scoliotic curve, compared to paraspinal muscle on the convex side, have enhanced anti-inflammatory

macrophage infiltration? If so, is this associated with fibro/adipogenic progenitor expansion and fibrosis?

Objectives:

Primary: To quantify macrophages and muscle inflammation in paraspinal muscle from concave and convex sides of the scoliotic curve

Secondary:

1. Profile chemokines in paraspinal muscle from concave and convex sides of the scoliotic curve.

2. Assess the role of fibro/adipogenic progenitor (FAP) in muscle fibrosis in AIS.

Hypothesis: In adolescents with AIS, enhanced anti-inflammatory macrophage infiltration of paraspinal muscle on the concave side, compared to the convex side of the curve, drives fibro/adipogenic progenitor expansion and differential fibrosis.

Methods & study procedures

Study design

This is a cross-sectional study. The participants will be recruited from Pediatric Orthopedic Clinic at McMaster Children's Hospital, a tertiary pediatric care center in Hamilton, Ontario, Canada. The study procedures are described in Figure 1.

Cohort characteristics

Inclusion criteria: We will include boys and girls, 10-17 years of age with a diagnosis of AIS confirmed on clinical and radiological grounds, and who have been informed by their team that they require and have agreed to undergo spinal surgery. We will include lean [BMI centile below 85th] and overweight/obese [BMI centile ≥85th] who are free from

infection for 15 days before sample collection.

Exclusion criteria: We will exclude patients with scoliosis related to other causes including congenital scoliosis, neuromuscular disorders, metabolic disorders, skeletal dysplasia, and syndromes. We will also exclude those on medications used within 15 days prior to sample collection (including high dose steroids, immunosuppressive therapy, and anti-thrombotic medications). We will also exclude those with active bacterial, viral or fungal infections, and chronic inflammatory diseases including autoimmune disease [Systemic Lupus Erythematosis, Juvenile Idiopathic Arthritis, Dermatomyositis]. We will also exclude smokers, pregnant girls and those who are unable or unwilling to provide signed consents.

Recruitment & consents:

The ICONS study will recruit patients who have been deemed eligible for scoliosis surgery. The healthcare provider will ask permission from potential participants to be approached by the study team. If patients and parents agree, the study team will meet the participants on the day of their preoperative evaluation to introduce the study and answer questions.

If the family and participant agree to participate, consent forms will be signed. The consent forms include those for parents and assent forms for children between 10-15 years of age. For those 16 years or older, the participants will sign their consents. Separate consent forms for genetic (DNA) testing are completed. The participants are assigned unique identifying numbers to protect confidentiality and data are anonymized shortly after collection.

Questionnaires:

After consent procedures are completed, study questionnaires are provided to the participants and their families.

The study will collect sociodemographic and clinical data including age, gender, grade in school, parental education, religion, ethnicity, history of medical or surgical problems, birth history, family income, social history, and family history of medical problems.

Regarding dietary information, the intake of different food items will be documented using a dietary questionnaire modified from the Adolescent Food Frequency Questionnaire(129, 130). Reported physical activity will be measured using the HAES questionnaire(131). Sleep will be measured using the Chevrin sleep questionnaire(132). We will also enquire about mental health by asking participants or parents to fill a questionnaire reporting mental health problems(133). Neighborhood walkability will be determined using the NEWS questionnaire(134).

Anthropometric & clinical measurements:

The participant will have their height measured closest to 0.1 cm using a stadiometer, weight to closest 0.1 kg using weighing scale, and Body Mass Index (BMI) in kg/m^2 will be calculated from height and weight and BMI centile determined from CDC growth charts. The waist circumference and hip circumference will be measured using a spring-loaded measuring tape closest to 0.1 cm. Puberty will be assessed using drawings describing Tanner staging for girls eight years or older, and external genitalia for boys nine years and older(135).

Sitting systolic and diastolic blood pressure (BP) will be measured twice using automated BP monitor, and pulse rate is measured using same device. Body fat percentage is measured

using Tanita body fat monitor for children (Tanita Corporation, Illinois, USA), and grip strength will be tested using a Dynamometer.

Biological samples:

All samples will be obtained on the day of surgery after an overnight fast of 8-10 hours.

Blood

Blood samples are obtained after patients are anesthetized and central lines are in place to permit blood sampling. We will be collecting serum and plasma samples as well as buffy coat for DNA studies. PAX RNA whole blood samples are also collected for RNA studies. Samples will be aliquoted to cryovials, and stored at -80 °C until further use.

Urine

Urine samples will be obtained using 90 ml urine containers after urinary catheterization for the surgical procedure. Urine is aliquoted to cryovials and stored at -80 °C until further use.

Tissue sampling and processing:

Paraspinal muscle (erector spinae) samples will be collected shortly after starting the surgical procedure for correction of scoliosis. The points of sampling include samples from the apex (maximal area of convexity) of the spinal curve, matched with a biopsy from the area of maximal concavity on the opposite side of the spinal curve.

Two important procedures are applied during sample collection that will ensure fidelity of immune cell phenotype. First, we will collect the samples before adrenaline injections, used to control bleeding during surgery, as it is known that adrenergic stimuli may change macrophage responses. We will also avoid cauterized areas of tissue sampling.

The muscle samples will be cleaned from blood by drying with gauze, and connective tissue is separated from samples. Tissue samples are then divided into five pieces, and four pieces will be snap frozen immediately in liquid nitrogen and transferred to -80 °C freezer for storage. One piece will be formalin-fixed and paraffin-embedded until further processing.

Experimental work details:

Primary objective: Determine the influence of curve sidedness on paraspinal muscle inflammation

Muscle macrophage content will be profiled using CD68 Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR), western blot and Immunohistochemistry (IHC)(136, 137).

For qRT-PCR, muscle tissue (25-50 mg) will be chipped and powdered, and then added to reagent. The tissue will be homogenized, and RNA isolation will take place using RNAeasy minikit (Qiagen).

One microgram of RNA will be used to generate cDNA utilizing SuperScript III reverse transcriptase kit (Invitrogen, Carlsbad, CA). Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) will be completed using TaqMan assay primer-probe mixes (Applied Biosystems) as we previously described(136, 138). Western blot and Immunohistochemistry will be performed as described previously by our group(136-138).

The full characterization of macrophage phenotype will be determined by testing markers of inflammatory macrophages including Cluster of Differentiation-11c (CD11c), Arginase II, and C-C chemokine receptor 2 (CCR2). Anti-inflammatory macrophages will be detected by using Cluster of Differentiation-206 (CD206), TGFβ1, and Cluster of Differentiation 301 (CD301) using qRT-PCR, western blot and IHC co-staining methods(139). We will use Nikon eclipse 90i microscope for imaging, and analyze images to determine macrophage content

using NIS Element 64 bit 3.22.11 Software (Nikon Inc., Melville, NY)(136, 137, 140).

To profile muscle inflammation, we will perform qRT-PCR using TaqMan assay primer-probe mixes (Applied Biosystems) on cDNA synthesized from RNA from muscle.
We will test the gene expression of pro-inflammatory TNFα, Interleukin-1β (IL-1β), Interleukin-6 (IL-6) and Interferon gamma (INFγ). Anti-inflammatory cytokines measured Include Interleukin-10 (IL-10) and Interleukin-1 receptor antagonist (IL-1Ra).
We will also measure cytokines in lysates from muscle (TNFα, IL-1β, IL-6, INFγ, IL-10, IL-1Ra) using Bio-Plex assays (Bio-Rad) as we previously reported(137).
Activation of inflammatory pathways responsible for cytokine & chemokine production will be tested using western blot including c-Jun-N-Terminal kinase (JNK), Extracellular signal-regulated kinase (ERK), p38 Mitogen Activated Protein Kinase (p38MAPK), and NFκB pathways (Cell signaling)(138).
Total and phosphorylated (activated) versions of the molecules will be quantified, and reported as a ratio of phosphorylated/total protein. GAPDH will be used as a loading control(138).

Secondary objectives:

1-Profile chemokines in paraspinal muscle from concave and convex sides of the scoliotic curve.

As chemokines drive macrophage migration to muscle, there is a need to determine the chemokines driving macrophage infiltration to muscle in AIS. We will assess the gene expression of CCL2, CCL3, CCL5, Macrophage Colony Stimulation Factor (MCSF), Granulocyte-Monocyte Colony Stimulation Factor (GM-CSF), and Chemokine (C-C) Ligand

20 (CCL20), which accounts for most muscle chemokinetic activity.

Chemokine protein content (CCL2, CCL3, CCL5, MCSF, GM-CSF, CCL20) will be assessed using Bio-Plex assays (Bio-Rad) as we previously reported(137).

2-Assess the role of fibro/adipogenic progenitor (FAP) in muscle fibrosis in AIS.

The experimental design of this part is described in Figure 2. We will generate macrophages and evaluate inflammatory (M1) and anti-inflammatory (M2) effects on FAPs. We will isolate FAPs from paraspinal muscle using $CD15^{+}PDGFR\alpha^{+}CD56^{-}$ markers after Collagenase digestion by flow cytometry(141).

To generate macrophages, we will differentiate THP-1 human monocyte cell line to macrophages (24 h incubation with 150 nM phorbol 12-myristate 13-acetate (PMA), followed by 24 h incubation in RPMI medium). Macrophages will then be polarized to M1 macrophages (20 ng/ml IFNγ+10 pg/ml LPS). Polarization to M2 will be done by incubating cells in 20 ng/ml IL-4+ 20 ng/ml IL-13)(142). We will collect the medium M1 and M2 cells are growing in. This conditioned medium from M1 (CMM1) and M2 (CMM2) will be used in this set of experiments.

To determine if inflammatory (M1) macrophages produce factors that reduce FAP proliferation and differentiation, we will treat FAPs with CMM1 for 24h. We will then measure FAP proliferation with BrdU incorporation assay, differentiation to adipocytes using oil red O staining, and differentiation to fibroblasts using gene expression and western blot of ER-TR7, FSP-1, and alpha smooth muscle actin. We will also test FAPs for apoptosis by performing TUNEL assay using TiterTACS® in situ kits (R&D Systems).

We will then measure CMM1 cytokines including TNFα using ELISA, and use TNFα neutralizer Adalimumab, and repeat FAP treatment(143). In addition, we will treat FAPs

with TNFα receptor antagonist (R-7050; Santa Cruz) (144) and repeat the experiments. To determine if anti-inflammatory (M2) macrophages produce factors that enhance FAP proliferation and differentiation, we will treat FAPs with CMM2 for 24h. We will then measure FAP proliferation with BrdU incorporation assay, differentiation to adipocytes using oil red O staining, differentiation to fibroblasts using gene expression and western blot of ER-TR7, FSP-1, and alpha smooth muscle actin, and apoptosis by performing TUNEL assay. We will then measure CMM2 cytokines including TGFβ, and then use TGFβ neutralizing antibodies and test FAP proliferation and differentiation. We will also treat FAPs with TGFβ receptor antagonist (LY2157299; Eli Lilly)(145), and repeat the FAP treatment with CMM2.

Statistical analysis:

Outcome variables will be summarized using descriptive summary measures expressed as mean (standard deviation) or median (minimum-maximum) for continuous variables, and number (%) for categorical variables. Data will be checked for normality using Kilmogorov-Smirnoff test, and log transformed if not Normally distributed. We will test differences in socio-demographic and clinical data between groups using Chi-square tests for categorical variables and T-test or Kruskall Wallis tests for continuous variables depending on the distribution. Statistical analysis of qRT-PCR experiments will be done using ΔΔCT(136). For ELISA, standard curves will be

generated and cytokines normalized to total protein content of the sample and reported as pg μL^{-1} μg^{-1} protein(137). For western blots, we will measure the total and phosphorylated protein and generate a ratio of total/phosphoprotein for comparisons (22).

For IHC, Macrophages will be counted manually at x400 magnification, and the number of macrophages is normalized to the section area. The researcher counting the cells will be blinded to the experimental group allocations as we previously reported (17).

For human samples, all analyses will be performed using regression analyses to pool and compare the groups adjusting for age, sex, BMI, ethnicity, and fitness. For in-vitro and murine studies, clinical data will be analyzed using T-test.

The results will be reported as estimates of the difference, corresponding 95% confidence intervals and associated p-values. Alpha is set at 0.05, adjusted using Bonferroni approach for multiple analyses. All analyses will be performed using SAS 9.2 (Cary, NC).

Sample Size: For sample size calculations for the full study, as macrophage content in paraspinal muscle has not been done in AIS before, power calculation is difficult. We aim to recruit 120 patients based on sample size calculations according to Norman & Streiner(146), whereby 10 patients are needed per marker studied. We plan to approach 200 patients, and assuming 80% recruitment rate, and 20% attrition, with one patient being evaluated weekly for surgery and 40 scoliosis surgeries per year at our institution, we plan to complete patient recruitment over 60 months for the full study.

Discussion:

Previous research has demonstrated the presence of fibrosis in paraspinal muscle in AIS(116). The immune system is one of the major players in muscle tissue remodeling via inflammation, yet this has not been studied in AIS.

This work will focus on understanding the mechanisms driving immune-muscle crosstalk in AIS, and the contribution of FAPs to muscle fibrosis. This work will aid the development of anti-inflammatory and anti-fibrotic therapies to help retain muscle strength, reduce pain, and delay or prevent the development of the scoliotic curve. This study may also reveal muscle-specific biomarkers that predict initiation and progression of the scoliotic curve, response to therapy, or the development of complications.

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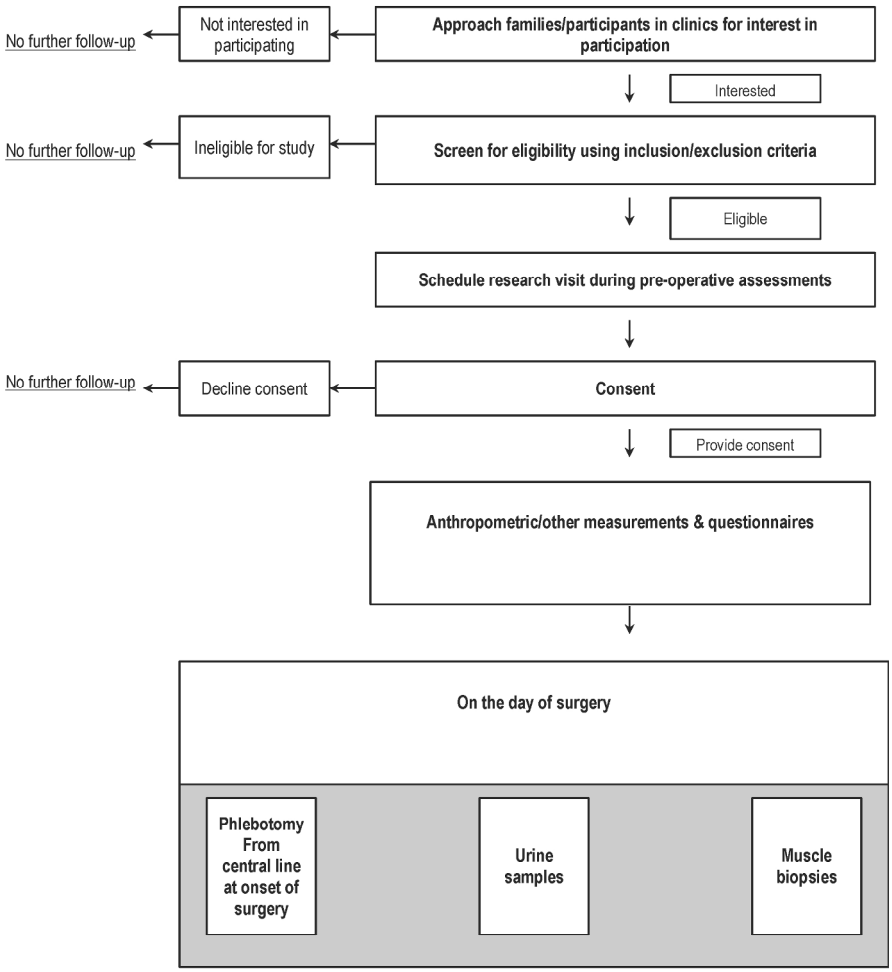

Figure 1: ICONS study flow diagram
215x279mm (300 x 300 DPI)

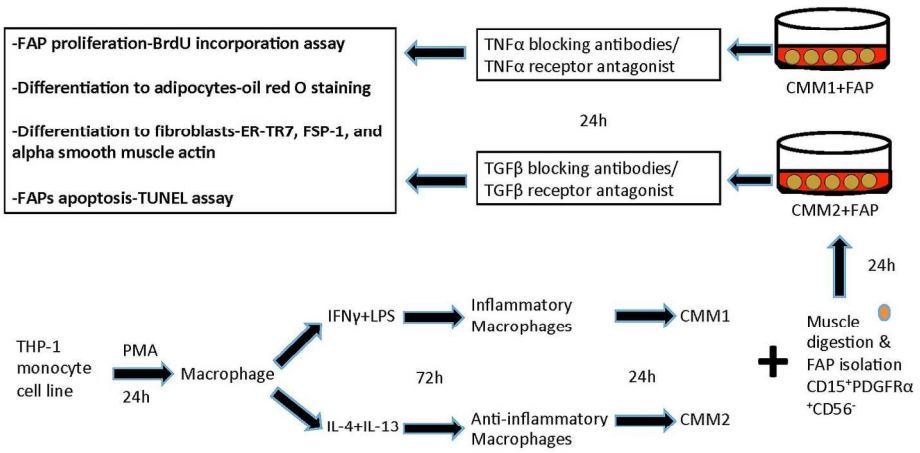

Figure 2: Testing macrophage-muscle crosstalk in AIS. THP-1 monocyte cell line is treated with 150 nM phorbol 12-myristate 13-acetate (PMA) for 24 hours to generate macrophages. Macrophages are treated with 20 ng/ml IFNγ+10 pg/ml LPS to generate inflammatory macrophages, and 20 ng/ml IL-4+ 20 ng/ml IL-13 to generate anti-inflammatory macrophages. The medium the cells are growing in is called conditioned medium (CM) for inflammatory (CMM1) and anti-inflammatory (CMM2) macrophages.
To generate fibro/adipogenic progenitors (FAPs), muscle will be digested with Collagenase, and FAPs will be plated into 12 well plates. We will then add CMM1 and CMM2 to FAPs, and then test FAP proliferation, differentiation to adipocytes and fibroblasts, and apoptosis.
156x92mm (300 x 300 DPI)