Université de Montréal

Étude de la mécanotransduction dans la scoliose idiopathique de l'adolescence (SIA)

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Ce mémoire intitulé :

Étude de la mécanotransduction dans la scoliose idiopathique de l'adolescence (SIA)

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RESUMÉ

À ce jour, la scoliose idiopathique de l'adolescent (SIA) est la déformation rachidienne la plus commune parmi les enfants. Il est bien connu dans le domaine de recherche sur la SIA que les forces mécaniques, en particulier les forces biomécaniques internes dans le système musculosquelettique, pourraient jouer un rôle majeur dans l'initiation et le développement de la maladie. Cependant, les connaissances sur la transformation des forces et des stimulations mécaniques en activité biochimique sont peu abondantes. Cet axe de recherche est très prometteur et peut nous fournir de nouvelles idées dans le dépistage et le traitement de la SIA. Dans le cadre de cette étude, nous visons à caractériser la mécanotransduction chez les patients atteints de la SIA en employant des techniques novatrices aux niveaux *in vivo* et *in vitro*.

Antérieurement dans notre laboratoire, nous avons démontré que les niveaux d'Ostéopontine (OPN) plasmatique chez l'humain corrèlent avec la progression et la sévérité de la maladie, et que ces changements sont observables avant le début de la scoliose. En plus, selon la littérature, l'OPN est une molécule sensible à la force mécanique, dont l'expression augmente en réponse dans de nombreux types de cellules chez plusieurs espèces. Toutefois, il n'existe aucune preuve que ce résultat soit valide *in vivo* chez l'humain.

L'hétérogénéité physique et biochimique de la SIA pose un gros défi aux chercheurs. Souvent, il est très difficile de trouver des résultats ayant une grande applicabilité. Les études portant sur les facteurs biomécaniques ne font pas exception à cette tendance. En dépit de tout cela, nous croyons qu'une approche basée sur l'observation des contraintes de cisaillement présentes dans le système musculosquelettique pourrait aider à surmonter ces difficultés. Les contraintes de cisaillement physiologique sont générées par des courants de fluide en mouvement à l'intérieur des os. Aussi, elles sont omniprésentes et universelles chez l'humain, peu importe l'âge, le sexe, la condition physique, etc., ce qui veut dire que l'étudier pourrait fort bien avancer nos connaissances en formant une base fondamentale avec laquelle on

pourra mieux comprendre les différences quant à la mécanotransduction chez les patients atteints de la SIA par rapport aux sujets sains.

Pour ce projet, donc, nous proposons l'hypothèse que les sujets atteints de la SIA se différencient par leurs réponses respectives à la force mécanique au niveau cellulaire (en termes de l'expression génique) ainsi qu'au niveau *in vivo* (en termes du marqueur OPN et son récepteur, sCD44).

Afin de vérifier la partie de notre hypothèse de recherche concernant l'aspect *in vivo*, nous avons recruté une cohorte de patients âgés de 9-17 ans, y compris i) des cas pré-chirurgicaux (angle de Cobb > 45°), ii) des cas modérément atteints (angle de Cobb 10-44°), iii) des témoins, et iv) des enfants asymptomatiques à risque de développer la scoliose (selon nos dépistages biochimiques et fonctionnels) d'âge et sexe appariés. Une pression pulsatile et dynamique avec une amplitude variant de 0-4 psi à 0.006 Hz a été appliquée à un des bras de chacun de nos sujets pour une durée de 90 minutes. Au tout début et à chaque intervalle de 30 minutes après l'initiation de la pression, un échantillon de sang a été prélevé, pour pouvoir surveiller les niveaux d'OPN et de sCD44 circulants chez les sujets. Nous avons découvert que le changement des niveaux d'OPN plasmatique, mais pas des niveaux de sCD44, corrélaient avec la sévérité de la difformité rachidienne chez les sujets, ceux ayant une courbe plus prononcée démontrant une ampleur de réponse moins élevée.

Pour vérifier la partie de notre hypothèse de recherche concernant la réponse mécanotransductive cellulaire, des ostéoblastes prélevées à 12 sujets ont été mis en culture pour utilisation avec notre appareil (le soi-disant « parallel plate flow chamber »), qui sert à fournir aux ostéoblastes le niveau de contraintes de cisaillement désiré, de manière contrôlée et prévisible. Les sujets étaient tous femelles, âgées de 11-17 ans ; les patients ayant déjà une scoliose possédaient une courbe diagnostiquée comme « double courbe majeure ». Une contrainte fluidique de cisaillement à 2 Pa, 0.5 Hz a été appliquée à chaque échantillon ostéoblastique pour une durée de 90 minutes. Les changements apportés à l'expression génique ont été mesurés et quantifiés par micropuce et qRT-PCR. En réponse à notre stimulation, nous avons trouvé qu'il n'y avait que quelques gènes

étant soit différentiellement exprimés, soit inchangés statistiquement dans tous les groupes expérimentaux atteints, en exhibant simultanément la condition contraire chez les témoins. Ces résultats mettent en évidence la grande diversité de la réponse mécanotransductive chez les patients comparés aux contrôles, ainsi qu'entre les sous-groupes fonctionnels de la SIA.

Globalement, cette œuvre pourrait contribuer au développement d'outils diagnostiques innovateurs pour identifier les enfants asymptomatiques à risque de développer une scoliose, et évaluer le risque de progression des patients en ayant une déjà. Aussi, dans les années à venir, les profils mécanotransductifs des patients pourraient s'avérer un facteur crucial à considérer cliniquement, particulièrement en concevant ou personnalisant des plans de traitements pour des personnes atteintes.

Mots clés : mécanotransduction, scoliose idiopathique, ostéopontine, contraintes de cisaillement fluidique, parallel plate flow chamber, biomécanique, sCD44, outils diagnostiques

ABSTRACT

Adolescent idiopathic scoliosis (AIS) is the most commonly occurring musculoskeletal deformity among children today. It is generally well accepted in scoliosis research that mechanical forces, especially the internal biomechanical forces of the musculoskeletal system, could well have a major role in the induction and pathogenesis of the disease. However, the process by which mechanical loads or stimuli are converted into biochemical activity (mechanotransduction) has not been explored so deeply. This emerging facet of research in AIS holds much promise for new insights into the disease. Here, we aim to characterize mechanotransduction in scoliosis patients using some novel techniques at both the *in vivo* and *in vitro* levels.

Previously in our lab, we demonstrated that the level of plasma osteopontin (OPN) and sCD44 in the human body is a strong indicator of disease progression and severity, and that these changes are observable before scoliosis onset. In the literature, OPN *in vitro* is known to be mechanosensitive, showing upregulation in response to mechanical stress in a variety of cell types across many species. However, to the best of the author's knowledge, no literature exists as to whether this behaviour carries over *in vivo* in humans.

A major difficulty in AIS research is the heterogeneity of the disease, both physically and biochemically. Because of this, many times it is difficult to find results with wide applicability to patients. Study of biomechanical factors in AIS is no exception. We believe, however, that study of fluid shear stress in the musculoskeletal system may be able to solve this problem for mechanotransduction-related issues in AIS. Native physiological fluid shear stresses in humans are experienced in the musculoskeletal system, caused by fluid movement over cells therein. These fluid shear stresses are omnipresent and universal in all humans, regardless of age, gender, fitness level, etc., which means that studying it could very well go a long way towards establishing a fundamental basis of understanding the differences as to mechanotransduction in scoliosis patients as opposed to normal cases.

In this project, then, we advanced the hypothesis that AIS patients are distinguishable in the way they respond to mechanical force at both the cellular level (in terms of gene expression) as well as globally at the *in vivo* level (in terms of the scoliosis marker OPN and its receptor sCD44).

To test the *in vivo* portion of our hypothesis, we recruited a cohort of patients between the ages of 9-17, each one of which fell into one of 4 subject groups: i) surgical cases (pre-surgery, Cobb angle > 45°), ii) moderately affected cases (Cobb angle 10-44°), iii) controls, or iv) asymptomatic children at risk of developing scoliosis matched for age and gender against healthy controls. A dynamic, pulsatile, compressive pressure of variable amplitude from 0-4 psi at 0.006 Hz was applied to the arm of each subject for a period of 90 minutes. Initially and at intervals of 30 minutes after the start of force application, blood samples were taken in order to monitor circulating plasma OPN and sCD44 levels in subjects. We found that the change of circulating OPN levels, but not sCD44 levels, measured *in vivo* in response to our mechanical stimulation was statistically significantly correlated to status of spinal deformity severity, with more severely affected subjects demonstrating lower magnitudes of ΔOPN.

To test the cellular portion of our hypothesis, osteoblasts from severely affected AIS patients and unaffected controls were cultured for use with our parallel plate flow chamber (PPFC) apparatus setup, which permits application of fluid shear stress patterns to cells in a predictable, controllable manner. Subjects were all females who fell into the 11-17 years age range, with scoliotic patients presenting with double major curves. A dynamic, sinusoidal and oscillatory fluid shear stress pattern was applied to osteoblasts at 2 Pa, 0.5 Hz for 90 minutes. Overall gene expression changes across RNA samples as a result of our stimulation were measured using microarray and qRT-PCR approaches. In response, only a very small number of genes are either mutually differentially expressed or statistically unchanged across all functional scoliotic subgroups while having the opposite condition in the control group, indicating a great degree of difference in terms of mechanotransductive response as compared internally between AIS functional subgroups, as well as between control and AIS patients.

Globally, this project's work may contribute to the development of innovative diagnostic tools to identify asymptomatic children at risk of developing scoliosis, and to assess the risk of curve progression at an early stage in those already affected. Also, in years to come, the mechanotransductive profile of a patient could be another integral factor to weigh, clinically, when considering or designing treatment plans for affected persons.

Key Words: mechanotransduction, idiopathic scoliosis, osteopontin, fluid shear stress, parallel plate flow chamber, biomechanical, sCD44, diagnostic tools

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List of Abbreviations

µL	microliter
ABR	Advanced Biomechanical Rehabilitation
AC	alternating current
AIS	adolescent idiopathic scoliosis
ALP	alkaline phosphatase
ANOVA	analysis of variance
APOLD1	apolipoprotein L domain containing 1
AT	25-hydroxy-16-ene-23-yne-D_3
BLAST	basic local alignment search tool
BMSC	bone marrow stromal cell
bp	base pair
C1S	complement component 1, s subcomponent
C57Bl/6j	C57 black 6
Ca	calcium
CALCA	calcitonin-related polypeptide alpha
CALM1	calmodulin gene 1
cAMP	cyclic adenosine monophosphate
cDNA	complementary DNA
CHD7	Chromodomain-helicase-DNA-binding protein 7
CHU	Centre Hospitalo-universitaire
CIHR	Canadian Institutes of Health and Research
CO_2	carbon dioxide
COX2	cyclooxygenase-2/prostaglandin E_2

Ct	comparative threshold
CX3CL1	chemokine (C-X3-C motif) ligand 1
CXCL1	chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)
CXCL2	chemokine (C-X-C motif) ligand 2
DAVID	Database for Annotation, Visualization and Integrated Discovery
DIO3	deiodinase, iodothyronine, type III
DMP1	dentin matrix protein 1
DNA	deoxyribonucleic acid
DUSP2	dual specificity phosphatase 2
E	Young's modulus
EDTA	Ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assays
ERK	extracellular signal-regulated kinase
EU	European Union
FBS	fetal bovine serum
FOS	v-fos FBJ murine osteosarcoma viral oncogene homolog
g	gram
GC	guanine-cytosine
G-protein	guanine nucleotide-binding proteins
h	height
HA	hyaluronan
HSD	honestly significant difference
Hz	Hertz
I	moment of inertia

IBL	Innovation Beyond Limits
IL-6	interleukin-6
IL-8	interleukin-8
IS	idiopathic scoliosis
IVD	intervertebral disc
JBMR	Journal of Bone and Mineral Research
JNK	c-Jun NH2-terminal kinase
JUNB	jun B proto-oncogene
kb	kilobase
KEGG	Kyoto Encyclopedia of Genes and Genomes
kJ	kilojoule
KLF10	Kruppel-like factor 10
L	liter
Lc	characteristic length
LLC	Limited Liability Company
MAP	mitogen activated protein
MAPK	mitogen activated protein kinase
MC3T3-E1	a line of mouse osteoblastic cells
MIAME	Minimum information about a microarray experiment
min	minute
ml	milliliter
mm	millimeter
mM	millimole
mRNA	messenger ribonucleic acid

N	constant allowing for end conditions
NCBI	National Centre for Biotechnology information
ng	nanogram
NOD	nucleotide oligomerization domain
OFF	oscillatory fluid flow
OPG	osteoprotegerin
OPN	osteopontin
P	passage number
Pa	Pascal
PBS	phosphate buffered saline
PDLIM5	PDZ and LIM domain 5
PFF	pulsatile fluid flow
PITX1	pituitary homeobox 1
PPFC	parallel plate flow chamber
psi	pounds per square inch
P-TEFb	Positive Transcription Elongation Factor b
Q	flow rate
qPCR	quantitative PCR
qRT-PCR	quantitative reverse-transcription polymerase chain reaction
RANKL	Receptor activator of nuclear factor kappa B ligand
Re	Reynolds Number
RIMBP3	RIMS binding protein 3
RIN	RNA integrity number
RMA	robust multiarray algorithm

RN7SK	7SK RNA
RNA	ribonucleic acid
ROS	reactive oxygen species
RUNX2	Runt-related transcription factor 2
s	second
sCD44	soluble CD44
sCD44std	sCD44 standard isoform
SD	standard deviation
SELS	selenoprotein S
SERPINB2	serpin peptidase inhibitor, clade B (ovalbumin), member 2
SFF	steady fluid flow
SHISA2	shisa homolog 2 (Xenopus laevis)
SIA	scoliose idiopathique de l'adolescence
SNP	single nucleotide polymorphism
TGFβ	transforming growth factor β
UK	United Kingdom
USA	United States of America
UV	ultraviolet
WNT	wingless integration site
α	Womersley parameter
α-MEM	α minimum essential medium
β/b	chamber width
Δ	delta or change in
μ	dynamic viscosity

µg	microgram
µL	microliter
ρ	density
τ	shear stress

Chapter 1 : Introduction

Adolescent Idiopathic Scoliosis is a disease of the spine that affects a significant proportion (1-3%) of young adolescents (by definition, between the ages of 10 years and 17 years, 11 months). In spite of the great diversity of ideas that have been pursued to this point, the ultimate etiopathogenesis of the disease continues to elude scientists. Nor is there a proven procedure at present that is capable of at least identifying those in the population at risk of developing scoliosis or of its progression in pre-existing cases. Several major avenues of research have been followed, such as genetics, growth hormone secretion, melatonin deficiency, and neurological mechanisms [1-4], to name just a few. However, one area of research that has not quite been as extensively followed in regards to AIS, and which could possibly underpin all of the aforementioned hypotheses of its etiopathogenesis, is that of mechanotransduction.

Mechanotransduction is the process by which external mechanical loads or stimuli are converted into biochemical activity [5]. With respect to AIS, it is well-established that a scoliotic spine and its surrounding musculature experience a very different stress and loading pattern than does a normal spine. We believe that these differential stresses and loads are capable of causing the genetic and biochemical changes in the spinal and musculoskeletal system of the body through mechanotransduction that lead to a scoliotic phenotype. But more interestingly, we also hope to delve into the possible detrimental mechanotransductive effects occurring due to forces and stresses experienced by non-spinal related parts of the musculoskeletal system. This may seem counter-intuitive indeed to some; yet, there do exist interesting examples of this occurring with respect to AIS. For instance, adolescents and children alike undergoing dental realignment through the use of braces to the teeth (but otherwise completely normal) experience the application of force to the jaw, which has absolutely no anatomical relation to anything in the spinal system. Yet, it has been shown in a previous study that these children tend to have a significantly higher incidence of scoliosis compared to normal populations [6].

Moreover, a broad-spectrum knowledge of exactly what genes and biochemical pathways are being altered through mechanotransduction must be elucidated. If this knowledge were ever to be acquired, it could well revolutionize the diagnosis and

treatment of AIS. Presently, at-risk persons are largely advised to take a passive “wait-and-see” approach for the very simple reason that there is no method to discriminate between those who will and will not develop scoliotic curves. Thus, one day, as a future application of this work, a diagnostic lab bench test developed on the basis of this knowledge gained as to mechanotransduction-induced genetic and biochemical changes may thus come to fruition. It would be quick and non-invasive, and enable a diagnosis of susceptibility risk to be made at a much earlier age, thus improving treatment options and patient outcome.

While it is not possible to provide an exhaustive review of all the burgeoning literature on mechanotransduction or the AIS disease, here in this first chapter we will attempt to give readers a measured perspective of the research in these areas and fuse the lot into a coherent whole in order to form appropriate background for presentation of the author’s research project on the subject.

Literature Review

1.1 Anatomy of the Vertebral Column

1.1.1 General Structure of the Spine and Constituent Vertebrae

Anatomically, the human spine is comprised of 4 major zones of vertebrae, starting in the neck with seven cervical vertebrae (C1 to C7), followed by twelve thoracic vertebrae (T1 to T12), five lumbar vertebrae (L1 to L5), terminating with the five sacrum and four coccyx vertebrae [7] as shown in **Figure 1.1**. A typical spinal vertebra consists of (1) a body and (2) a vertebral arch, which has several processes (articular, transverse, and spinous) for articular and muscular attachments [8]. Between the body and the arch is the vertebral foramen: the sum of the vertebral foramina constitutes the vertebral canal, which houses the spinal cord. The vertebral arch consists of right and left pedicles (which connect it to the body) and right and left laminae. The vertebral arches are connected by ligaments, particularly strong in the lumbar region, e.g., the ligamenta flava between the laminae. The spinous processes are united by the interspinous and supraspinous ligaments, which merge in the neck with the ligamentum nuchae. This latter ligament is a median partition between the muscles of the two sides of the neck and is attached to the occipital bone. The transverse processes emerge laterally at the junction of the pedicles and laminae, and the spinous process proceeds posteriorly from the union of the laminae. The superior and inferior articular processes project vertically from the vertebral arches on each side and bear articular facets. When vertebra are in their anatomical position, notches between adjacent pedicles form intervertebral foramina, each of which typically transmitsneural structures including a spinal ganglion and a ventral root of a spinal nerve [9-11].

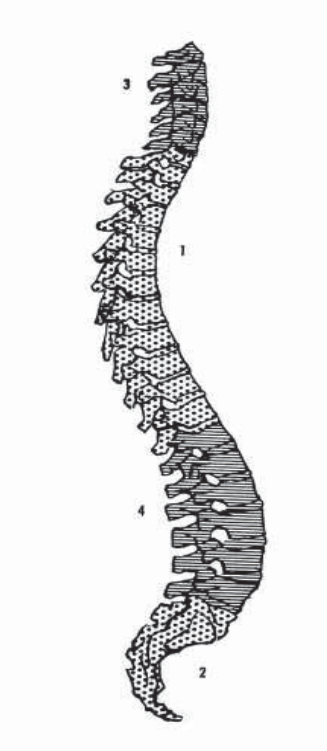

Figure 1.1 The four vertebral zones of the human spine: 1, thoracic; 2, sacral/coccyx; 3, cervical; 4, lumbar. Adapted from [7].

1.1.2 Intervertebral Disc (IVD)

Between each vertebra lies an intervertebral disc which is in constant contact with adjacent vertebral bodies via the hyaline cartilage endplates, and whose body is mainly spongy red marrow (**Figure 1.2**) [12]. The IVD has three main functions: a) maintain the linkage between vertebrae; b) act as shock-absorbers for the spine; and c) permit mobility and flexibility of the spinal column entity [13]. The intervertebral discs account for about a quarter of the length of the vertebral column. Each disc consists of a semi-gelatinous nucleus, called the nucleus pulposus, surrounded peripherally by the anulus fibrosus. The anulus fibrosis consists of fibrocartilage containing concentric layers of dense, regular connective tissue. In these layers, the collagenous fibers in any given annular layer are oriented nearly at right angles to the adjacent layers. Overall, the discs contain much water, diminution of which (temporarily during the day and permanently in advanced age) results in a slight decrease in stature. The fluid nature of the nucleus acts as a hydraulic spacer to maintain the height of disc [14].

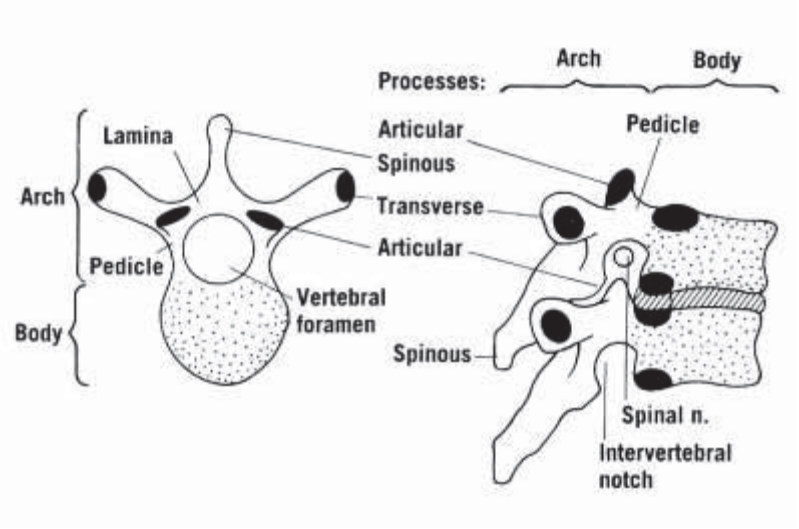

Figure 1.2 Parts of a vertebra as seen from above (left) and from the side (right). Adapted from [7].

1.1.3 Surrounding Musculature of the Human Vertebral Column

In terms of muscle control of the vertebral column, its chief muscular flexors are the prevertebral muscles, recti abdominis, iliopsoas, scaleni, and stemomastoids. Normally, the muscles of the back are relatively inactive when one is standing at ease. Gravitationally-induced movement is controlled by the erector spinae muscles, which also serve as the chief extensors. Lateral flexion is carried out mainly by the oblique muscles of one side of the abdominal wall. Injury or inflammation may easily result in reflex spasm of the muscles of the back [7, 15].

1.2 Adolescent Idiopathic Scoliosis (AIS) Fundamentals

1.2.1 General Overview

AIS is a three-dimensional structural deformity of the spinal column where the Cobb angle is greater or equal to 10 degrees, by definition affecting children aged 10-17 [16]. The three-dimensional aspect refers to the fact that the deformity can be some sort of rotation/curvature in the sagittal, coronal, and/or transverse anatomical planes, while the appellation of the term idiopathic to the disease indicates the absence of any known discernible cause for the deformity. Generally speaking, there are four basic scoliosis curve types, as shown in **Figure 1.3**: thoracic, lumbar, thoracolumbar, and double major. More complex curve patterns that exist are built from some combination of these elementary forms. The frequencies of occurrence of each of these four basic scoliosis

curve patterns are not equal. Thoracic and double major curves by far are the more commonly found. A representative Saudi Arabian clinical study reported that in their cohort of 359 patients, thoracic and double major curves accounted for 46% and 36% of all those classified [17].

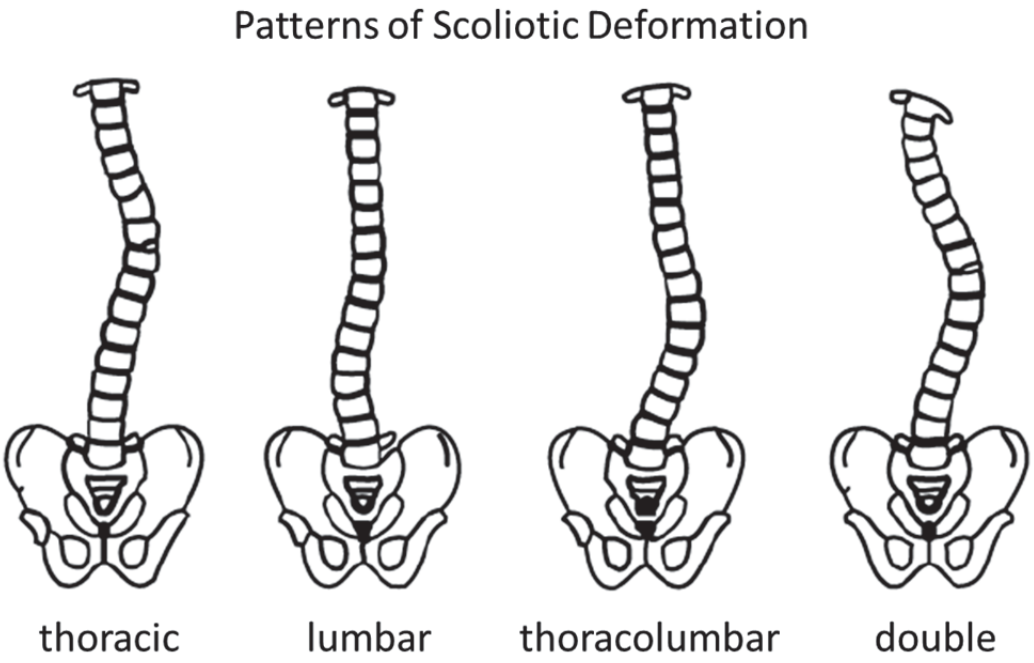

Figure 1.3 Four basic scoliotic curve types. Adapted from [18].

According to some studies, the average scoliosis patient will suffer a 14-year reduction in their average life expectancy [19]. During that lifespan, many patients also experience negative secondary effects on overall quality of life arising out of AIS affliction, such as reduced physical capability [20], impaired respiratory and/or cardiovascular function in more severe cases [21], and even psychological damage in terms of the patients' perceived well-being and self-image [22]. Worldwide, the cost impact to the healthcare system of the scoliosis disease itself is enormous. For example, in the United States alone, the burden of treatments and hospitalization are estimated to cost the healthcare system in excess of 10 billion dollars per year [23]; an average hospitalized scoliosis patient bears an average of $120 000 of out-of-pocket expenses for treatments [24].

1.2.2 Prevalence/Incidence

Prevalence, defined as the proportion of diseased individuals in a given population at a particular time point, and incidence, defined as the rate of new cases per time period in a given population, are two epidemiological measures of disease penetrance [25]. AIS is the most common spinal deformity in the world, affecting around 1-3% of the adolescent population [16], with a significant gender bias in favour of females. The prevalence of AIS in females is roughly 5-10 times greater than in males [26, 27]. The reason for this disparity is not known for certain. As well, in the US alone, every year there are roughly 173 000 new cases identified [19].

1.2.3 Risk Factors

There are a number of risk factors associated with the probability of AIS development in an individual. First and foremost, there definitely seems to be a genetic heritability factor; that scoliosis tends to be more common within affected families suggests this [1]. Reports of twin studies have also supported the genetic basis of AIS [28]. However, zeroing in on particular genes definitively conferring AIS risk has proven a challenge. Recent tries have attempted to advance the occurrence of polymorphisms in CHD7 [29] and estrogen receptor genes [30] as causative agents, but their real level of contribution as risk factors remains hazy at best. Certain environmental and lifestyle factors have also been hypothesized to increase risk of AIS. Indeed, strongly elevated prevalence of AIS in study populations among young people who participate actively in activities such as ballet [31, 32], gymnastics [33, 34], or playing musical instruments [35] (including one celebrated case [36]) has been observed. In our own lab too, we have noted that exposure to certain mycobacteria (during water-related activities like swimming) and selenium deficiency can raise the risk of developing scoliosis.

1.2.4 Aetiology

Though much debated, the precise etiopathogenesis of the disease is still uncertain to modern researchers. Some examples of foundational theories were stated in the introduction to this chapter. But it would be a monumental and likely impossible task to attempt to recapitulate the entire body and breadth of research upon this topic heretofore conducted. Ideas abound in the quest for a unified theory of AIS etiology and

pathogenesis; their range and diversity seem constrained only by the creativity and imagination of the scientists who conceive them. This is easy to see when we observe that even such ostensibly *outré* factors like the concentration of trace elements (e.g. zinc, copper, selenium, lead) in the bodies of AIS patients [37-40] or the seemingly innocuous everyday habit of wearing a backpack [41-43] have ardent followings in the AIS etiological research community. Suffice it to say, however, the most topical major subgroupings of modern ideological notions concerning the etiopathogenesis of AIS include but are not limited to: genetics, hormone/metabolic dysfunction (principally estrogens), melatonin, calmodulin, neurological abnormalities, biomechanical factors (including abnormal growth and development) [44-47]. We have already briefly touched upon the manner in which genetics may influence AIS development. As well, since the influence of biomechanical factors in AIS is a critical ingredient to the substance of this work, this matter shall be treated in detail in a separate section of this review below. For now in this section, then, we restrict our attention to the remaining subcategories, each to be covered in their turn.

1.2.4.1 AIS Aetiology: Estrogens

Recent discoveries in the pathogenesis of adolescent idiopathic scoliosis (AIS) suggest that estrogens have a role in its onset and development. Certainly such a relationship is intuitive, given the AIS affected state bias towards females, as previously mentioned. Idiopathic scoliosis is a disease whose occurrence frequently coincides with puberty, a stage when estrogens play an especially large part in growth and development. It is well known that estrogens have an active role in bone remodeling [48], a process that increases during puberty [49]. Several studies have found that estrogens and estrogen antagonists (17-α-ethynylestradiol) directly impact the development of scoliosis in animal models [50, 51]. Estrogens also probably do interact with growth hormones and growth factors, both of which are also considered potential etiological factors in AIS. Scoliotic individuals tend, on average, to be taller than their non-scoliotic peers [52], which could be explained by increased circulating growth hormone levels [53]. However, despite all this, there is no direct connection proven as yet between estrogen signaling and the progression of scoliosis in humans.

1.2.4.2 AIS Aetiology: Melatonin

In the human body, melatonin is a hormone that has important roles in maintenance of circadian rhythms [54]. In relation to scoliosis, previous studies in which chickens, rats, and hamsters were pinealectomized [55] and thus had melatonin production capability compromised showed a steep increase in scoliosis among the populations. However, melatonin-replacement therapy in pinealectomized animals seemed to have no beneficial effect on the scoliosis in many of these animals [56], nor did pineal gland transplantation [57], interestingly enough. Then in 2002, Nette et al. provided evidence of a possible serum threshold value of melatonin necessary for scoliotic development when they exposed normal and pinealectomized chickens to constant 24 hour/day sunlight to remove any possible artifacts from pinealectomic surgery [58]. In both experimental groups, there was a significant increase in the percentages of scoliotic chickens, from 0% to 15% in the normal chickens, and from 50% to an astounding 80% in the pinealectomized group. In our own laboratory, we have been studying and observing some interesting possibilities that melatonin may have effects as a molecular determinant in AIS [59] and possible utility as a molecular classifier for scoliotic patients into functional groups, based on differences observed at the G-protein level in the melatonin signalling pathway [60].

1.2.4.3 AIS Aetiology: Calmodulin

Calmodulin is a target that has regained scientific interest in recent years as well. As the name perhaps implies, it is a protein with a high capacity and specificity for binding calcium. Calmodulin serves as an intracellular Ca^{2+}-receptor and mediates the Ca^{2+} regulation of cyclic nucleotide and glycogen metabolism, secretion, motility and Ca^{2+} transport [61]. It is perhaps most appropriate to bring it to attention here, since calmodulin happens to be a second messenger of none other than melatonin, and is effective in regulating its release [62, 63]. In regards to scoliosis, calmodulin on its own has been on the scientific radar at least since the mid-1980s, as it was discovered that alterations of calmodulin activities in platelets of AIS patients were arising [64]. However, the interest of AIS researchers in the molecule appears to have waxed and waned sporadically over the course of time. It was not until roughly 10 years later that Kindsfater

revived the thread of study, finding that based on a single determination for each patient during growth, platelet calmodulin levels were higher in skeletally immature patients with progressive curves (10° per year) than those with non-progressive curves and age-matched controls [65]. The problem was that this discovery, promising as it was clinically, was based on a very small cohort; when extended into a more comprehensive longitudinal study, the trend broke down somewhat [66] (an independent study performed just two years ago even flatly contradicted Kindsfater's outcome in platelets [67]). In addition for Kindsfater, there was an inexplicable discrepancy between baseline levels of different subjects in the patient series that did not allow for development of a normal range for platelet calmodulin. Even to this day, no normal range for platelet calmodulin has ever been established [68], making the result impossible to exploit from a clinical perspective, for ends such as development of a predictive diagnostic screening tool. Evidence from the longitudinal study published in 2002 did suggest, however, that raised concentrations of calmodulin in platelets could result in altered skeletal muscle activity and subsequent progressive curvatures. Now, after another relative lull, interest in calmodulin as a scoliosis target is flourishing once again, with a spate of papers coming out in 2009. That year, at the genetic level, a Chinese group found certain single nucleotide polymorphisms (SNPs) of a calmodulin-encoding gene, CALM1, having some association to certain progressive subtypes of AIS, particularly the double major curve [69, 70]. More remarkable from a fundamental molecular point of view in AIS etiological research, however, was the set of articles exploring the possibility of using drug antagonists to decrease scoliotic curve severity and progression, with positive results [62, 71, 72]. The curious feature of note is that these works employed animal models traditionally seen in melatonin-deficiency studies of AIS, the pinealectomized chicken and bipedal C57Bl/6j mouse models; given what was just said about the close connection between calmodulin and melatonin, perhaps this might imply some kind of axis or interaction between the two at work. Of course, the applicability to humans remains in question, since one must bear in mind that these *are* still simply animal models.

1.2.4.4 AIS Aetiology: Neurological Mechanisms

Given that the spinal cord is undeniably a centrepiece in the entire human nervous system, an organ system with incontrovertible functions in individual growth and development, it stands to reason that this might well lead some to speculate theories implicating neurological involvement in the ultimate development of its structural dysfunction. Indeed, over the last thirty years, sophisticated neurological investigations have been used to compare patients who have idiopathic scoliosis with controls and to compare patients who have progressive curves with those who have stable scoliotic curves [73, 74]. Unfortunately, results have mostly been inconsistent [75]. Moreover, no definite neurological test either for diagnosing AIS or to predict risk of progression has come to fruition from these efforts as yet.

Early attempts to implicate neurological system functions in AIS pathogenesis generally were directed towards attempting to prove inequalities in vibration sensing and proprioception in AIS patients versus controls that could lead to the scoliotic state [76, 77]. Evaluation of vibration sensitivity was typically done by means of a biothesiometer, but the results were generally erratic between studies, and the biothesiometer itself has since been deemed unreliable [78]. Similarly, impaired peripheral proprioception is not a constant finding [79]. Interestingly, however, AIS patients do consistently experience difficulties with spatial orientation as opposed to controls [80], which may be explainable in terms of contemporary work showing cerebral cortex alterations in AIS patients localized to regions chiefly responsible for motor and vestibular functions [81].

Overall of late, the chief polemic for those championing neurological involvement in scoliosis development seems to be Burwell. His chief contribution has been to advance a grand hypothesis of AIS etiology based on a so-called precept of “asynchronous neuro-osseous” growth [82]. This theory is illustrated and summarized in **Figures 1.4** and **1.5**. In short, curve initiation may occur because in puberty, the hypothalamus of those susceptible to AIS exhibits selectively increased sensitivity to circulating leptin, whose levels tend to rise dramatically during this period, leading to asymmetry [83]. Concurrently, the maturation of the somatic nervous system, responsible for postural integrity of the spine, is delayed relative to its autonomic counterpart [84]. This may be because the pubertal growth spurt is so sudden, and the spinal cord simply grows

physically at such an incredible rate, or because the maturation of the somatic system is retarded due to abnormalities in afferent, central, or motor mechanisms [85]. Whatever the case, this decoupling between the relative rates of growth and development of the autonomic and somatic components of the nervous system leads to progression, according to this scheme, since the postural mechanisms regulated by the somatic system just do not have the capacity to regulate skeletons of such size and are thus unable to check the growth of the initiated curve. The theory's link with leptin would explain the predilection of AIS to predominantly affect females, for girls on average have higher serum leptin levels before, during, and after puberty than boys [86]. On the down side, Burwell's elaborate model, though elegant, does not account for AIS cases that could arise due to abnormalities in the brain [82, 87-89].

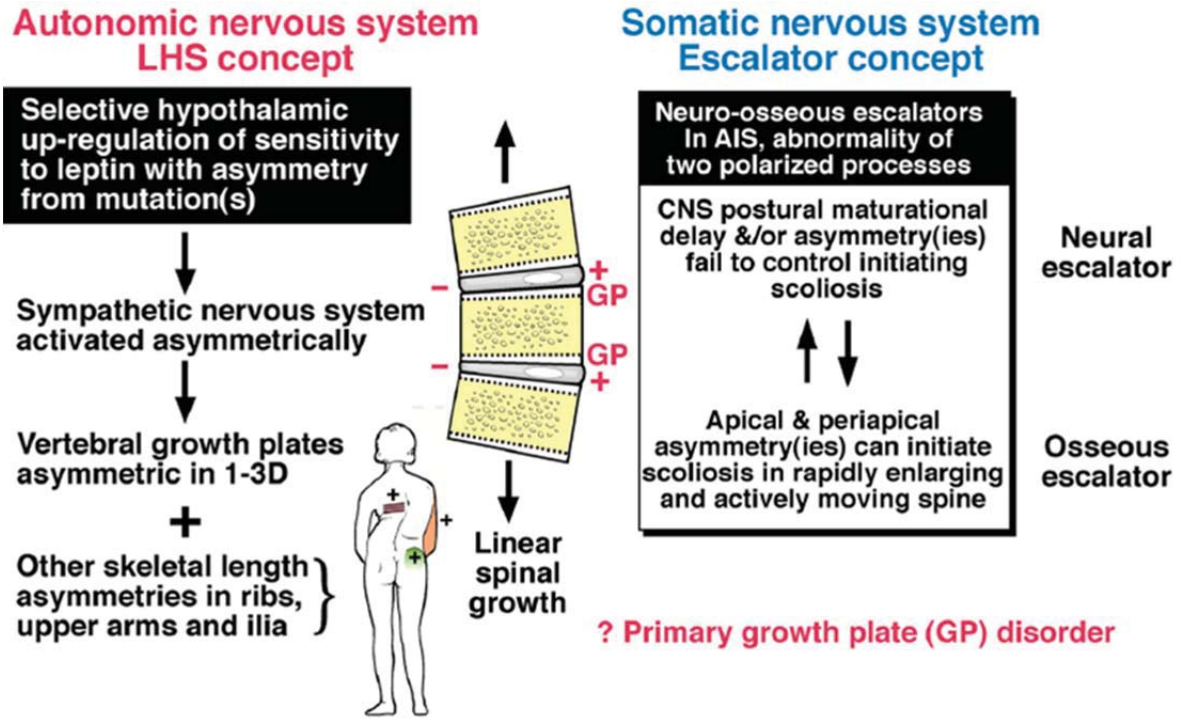

Figure 1.4 Events in the autonomic and somatic nervous systems according to Burwell's theory of asynchronous neuro-osseous growth leading to AIS pathogenesis. Adapted from [4].

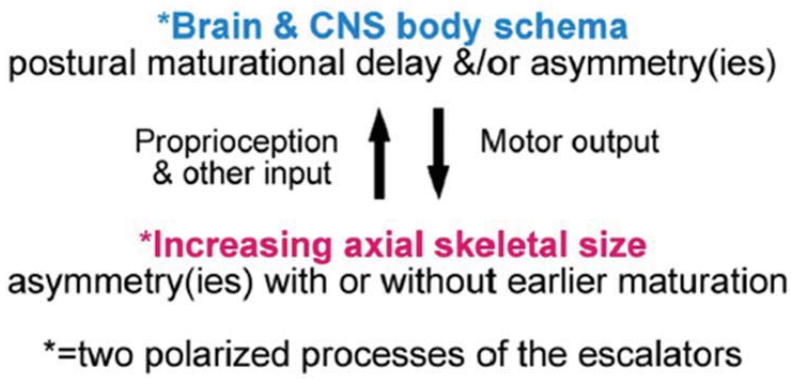

Figure 1.5 Maturational delay in Burwell's theory of AIS pathogenesis. Due to this delay, the somatic nervous system is unable to regulate the skeleton, leading to induction/progression of curves.

1.2.5 Diagnostics

Diagnosis of AIS is one of exclusion, made only when it is certain that the curvature is not due to other agents such as vertebral malformation, neuromuscular degeneration, or syndromes having scoliosis as a symptom [16]. Patients are generally first screened clinically using a scoliometer and Adams' forward bending test; however, a definite diagnosis cannot be concluded until a radiological measurement of the spinal curve returns a Cobb angle value of not less than 10 degrees on a standing coronal X-ray [90]. By definition, the Cobb angle is the angle between the upper border of the uppermost vertebra in the scoliotic curve and the lower limit of the lowermost [91]. Despite the number of other procedures over the years that have been put forward with the idea of improving upon the accuracy of scoliosis assessments [92-95], the Cobb angle remains the gold standard for curve quantification. Patients are thus typically classed by curve severity (i.e. Cobb angle) and location. Curves of magnitude between 10-45° are widely accepted as being "moderate" in severity, while those greater than or equal to 45° are considered to be "severe" [96]. Curves less than 10° are "mild" or negligible and not treated as being scoliotic. Curve location is specified as mentioned before.

The problem with these existing standard methods, even accounting the aforementioned attempts at "improvement", is that while they are simple in their execution, they are also only capable of identifying individuals *ex post facto*. They do not respond at all to the more important and demanding question of identifying those whose existing curves will progressively become more pronounced, thus potentially requiring

intervention, or those who are asymptomatic at a given moment but who have a strong probability of developing a severe curve in the future. This is extremely dangerous, as AIS is a highly fluid disease, and thus so is any "prognostication" of a particular moment. That is to say, though a young individual may present with only a slight or negligible curve at a particular screening occasion, they can increase rapidly during adolescent growth spurts. Reports have indicated that during puberty, curves are capable of progressing as much as 10° or more in a year [91]. However, there is no proven method or test available to prophylactically identify children or adolescents with such a propensity. The typical *modus operandi* then, is to adopt a "wait-and-see" approach until a significant deformity or progression is demonstrated, by which time the best window of opportunity for intervention may already have passed. This is unfortunate and represents a significant challenge for clinicians in scoliosis care today, as only with earlier identification and intervention can more detrimental consequences of progressive AIS be avoided.

As we saw in the previous section, there has been some debate as to whether or not measurement of platelet calmodulin levels might offer some hope for supplying missing predictive value, but obstacles were encountered that have yet to be resolved. In our laboratory, we ourselves have attempted to improve this situation, recently developing a predictive blood test based on measuring circulating levels of osteopontin (OPN) and soluble CD44 (sCD44) in plasma (more on these molecules later), two markers that we have demonstrated to be strongly associated with AIS risk [97, 98], in order to pre-emptively identify those at risk of development or progression of curves. However, while this is a very positive step, this "diagnostic test" still spans a period of 18 months before arriving at an answer, far too long for wide clinical usefulness as yet. More work is needed in this regard.

1.2.6 Treatments

Whereas the identification and diagnosis of an already-present scoliotic curve is usually relatively straightforward and perhaps even self-evident in most cases, the question of what to do about it has been a vexed question to clinicians. First, although there are general guidelines for treating IS, each patient and each curve is different, and even curves of very similar configurations and magnitudes may demonstrate very

different tendencies of progression or cause significantly different symptoms. These differences preclude universal procedures for treating scoliosis, leaving the actual course of treatment largely dependent on the judgement and discretion of the particular attending physician. In general, however, as AIS is by its very definition a malady occurring before skeletal maturity, there are some commonalities in approach and precautions taken in attempted treatments.

For most clinicians, primarily because of concerns of progression before completion of skeletal growth, as well as the fact that the more dramatic treatment options at an orthopaedist's disposal always entail consequences that cannot be undone, conservative options are almost always favoured whenever possible. The decision to treat or not is typically based on an evaluation of risk of progression, and if applicable, the presence of serious secondary effects stemming from curvature. In the latter case, these mostly present in only the most severe scoliosis curvatures, to whose manifestations we have already alluded (e.g. cardiopulmonary dysfunction, respiratory difficulty, etc. due to reduced space in the thoracic cavity). Here, the decision to treat is quite a simple and necessary one to take. For the rest, however, a period of considered observation and examination of the patient's spine through radiological measurement, spanning many months, sometimes years, is normally first undertaken before all else [16]. This time period allows the clinician to accumulate data in order to help gauge the rate of change of curvature, and thus the risk of curve progression. The observational period, alas, forms but one aspect of the progression risk assessment. Other factors taken into account in this assessment include maturity (age at diagnosis, menarchal status for females, skeletal growth remaining), family history of AIS, and size and position of the curve apex. The more skeletally immature the patient is, the greater the probability of progression in many cases [99-101]. In addition, larger curves at time of clinical presentation involve higher likelyhoods of progression [102]. Finally, certain curve types have higher prevalences of progression than others; for instance, thoracic curves have had progression rates reported anywhere from 58% [100] to 100% [102] in certain study populations. Double curves have a greater chance of progression than single curves [103].

Regardless of specifics, for those patients whose evaluation reaches a point where, in the clinician's judgement, an intervention is necessary, treatments branch into two families, bracing and spinal surgery. Bracing is the prescribed wearing of an external orthosis around the torso, indicated for children still in the "moderate" severity of classification, especially those with significant skeletal growth still to come. There exist numerous options in terms of braces, of which some examples are the Milwaukee [104], Boston [105], Charleston bending[106], as well as the newer SpineCor braces [107]; however, the goal remains the same: stabilize or halt altogether the progression of the curve. Surgery is the most drastic action to be taken, normally the very last recourse for treatment. It is employed for "severe" curves and where bracing has failed to produce the desired effect. Surgical intervention typically aims to abruptly halt progression and also physically straighten the curvature of the spine as much as possible. The gold standard of such intervention is spinal fusion with instrumentation [108]. A sample X-ray showing a spine that has undergone this procedure is shown in **Figure 1.6**. In this procedure, bone is grafted to the vertebrae so that when it heals they will form one solid bone mass and the vertebral column becomes rigid. The procedure can be performed from the anterior (front) aspect of the spine by entering the thoracic or abdominal cavity or, more commonly, from the back (posterior). In more severe cases, both are employed. Although levels of curve correction can be quite high in this scheme, it is not without drawbacks. First, though this prevents worsening of the curve, it comes at the expense of some spinal movement and a good deal of remaining spinal growth potential [109]. Also, back pain, where present, is not always improved by surgical correction, and may even be worse afterwards [110]. Finally, instrumentation failure is not uncommon. A 2005 Japanese study reported instrumentation corrosion after long-term use in roughly two-thirds of its cohort, which can cause heavy metal toxicity, diminished immune response, and chronic inflammation [111].

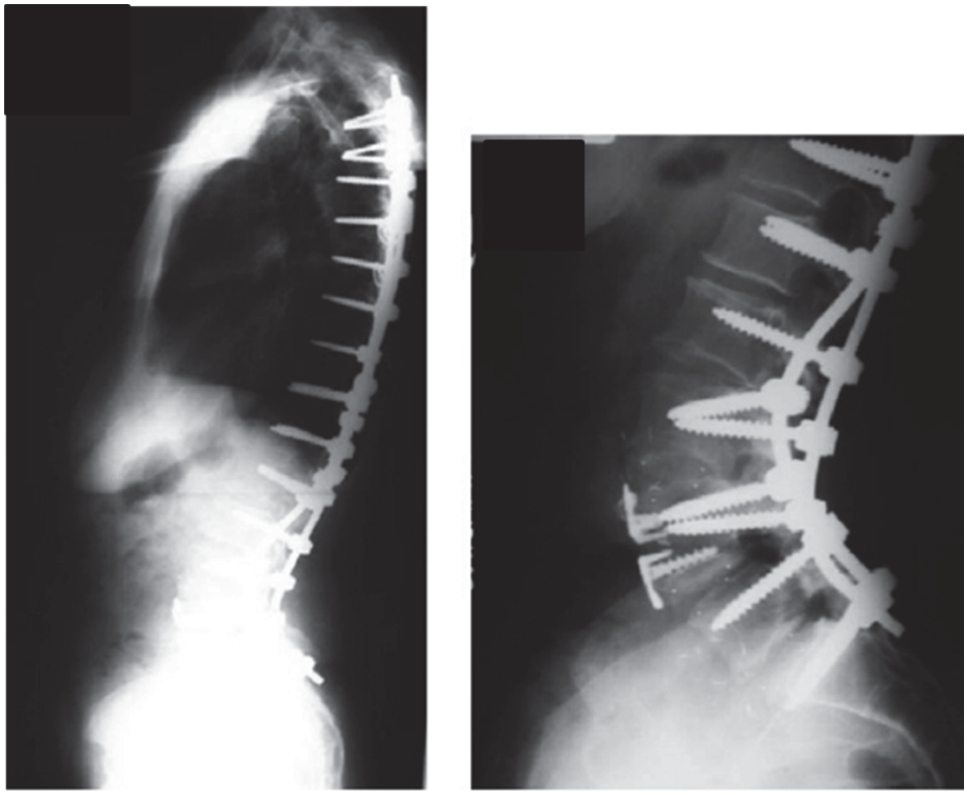

Figure 1.6 X-ray of a spine that has undergone an instrumentation + fusion procedure. Adapted from [90].

1.3 Osteopontin (OPN) Basics

OPN is an O-glycosylated phosphoprotein synthesized locally in a variety of cells and tissues that is also capable of existence in circulating serum of the body [112]. Also called a bone sialoprotein, it was originally identified as a bone matrix protein [113, 114], and later as a cytokine [115], and a key player in biomineralization [116]. Roughly 300 amino acids in length and possessing a highly negative charge, its most commonly bound receptor is one known as the CD44 receptor family [117], although a recent study showed that osteopontin did not interact with the most common CD44 isoforms [118]. It is found at limited levels in healthy humans and animals, with the bones and kidneys having the greatest overall content [119], although many epithelial cells and bodily secretions like urine and saliva also contain it. Most well-established and explored is its role in human immunity [120], in addition to a host of pathologies. The immunological and pathological situations are connected by way of a common inflammatory component, and indeed, OPN has been cited a number of times as a key signalling molecule in the inflammatory response [114, 116, 121].

The OPN signaling pathways are not well understood, although besides interacting with CD44 receptors at the cell surface, OPN can interact with many integrins, particularly the $\alpha_v\beta_3$ variety, as well as Toll-like receptors [122]. There exists only a small body of relevant literature at this point as regards possible modulators and interacting partners of OPN that can produce changes in its post-translational status. However, knowledge of OPN's possible post-translational status changes are of interest as it is possible that these changes might have a profound effect on its functional capabilities in vivo. As an example, OPN upregulation has been observed profoundly so not only in scoliotic patients in a variety of tumour cells and sites, cardiovascular myopathic diseases, liver disease, asthma, multiple sclerosis, even psoriasis [112, 115, 118, 120, 123]. With so many associated pathologies linking with OPN, each one distinctly unrelated phenotypically with another, it seems intuitively obvious that there is more at work in these conditions than simple inappropriate OPN activation to explain them. Post-translational changes to OPN may thus be another portion of the explanation.

Upregulation of OPN through Vitamin D uptake in vitro was already known in 1994 from a study by Khoury's team on osteoblast-like ROS 17/2.8 cells [124]. But in 1998 Vitamin D was found to have a modulatory effect on OPN post-translational status by shifting the produced isoform's isoelectric point upwards (a post-translational effect) by Safran et al. [125]. Not only was the charge form of OPN different, however, but Safran also tested for and observed a change (reduction) in the phosphorylation level of the new isoform, suggestive of a more molecularly active OPN. Interestingly, the phosphorylation could be mimicked by analog AT (25-hydroxy-16-ene-23-yne-D_3) which is known to trigger Ca^{2+} influx in cells or inhibited by Ca^{2+} channel blockers, indicating dependence of the signalling on Ca^{2+} influx [126].

This idea of a link between [Ca2+] and OPN has been strengthened recently. Calcium ions were illustrated as being essential to OPN expression by Wu et al. in 2003 [127]. Further confirmation was obtained by in abolishing OPN expression in cultured MC3T3-E1 osteoblasts by application of thapsigargin, a calcium ion channel blocker [128]. These findings are significant as calcium ion signalling represents one of the principal cellular responses to physiological stress [129]. In signal transduction, it can act through

its own specialized ion channels or indirectly as a second messenger system like in G-protein signalling. Moreover, as calcium ion flux is one of the first cellular actions to take place in response to mechanical stresses, this hints at another facet of OPN that we will now examine: its role in mechanotransductive response.

1.4 OPN in mechanotransduction

In the musculoskeletal system, OPN is not normally expressed in the mechanosensing-capable cells (the osteoblasts and osteocytes) of healthy animals [130, 131]. There is a growing body of evidence, however, that indicates its potentially potent role as a mediator of mechanically-induced responses in the musculoskeletal system. Independent pilot studies in 1999 first connected OPN with mechanically-based signalling; interestingly, these were done in anatomically unrelated parts of the body, suggesting that OPN has a response capability that is system-wide in the body. First, Miles showed that the tibiae of adult female Sprague-Dawley rats that had received a typical four-point bending test experienced an almost 4-fold increase in OPN mRNA levels [119]. Terai's group, on the other hand, though studying orthodontics and tooth movement, also noted an astounding OPN response in observing a dramatic increase in the number and proportion of osteocytes expressing OPN in the jaws of male Sprague-Dawley rats subjected to an experimental tooth movement model [132]. These convictions were further strengthened when similar increases in OPN mRNA levels were confirmed by Carvalho in embryonic chicken calvarial osteoblast cells, who incidentally also demonstrated that such induction of expression is likely mediated through integrin receptors [133].

Is OPN expression truly critical in the musculoskeletal system for normal function? Chellaiah et al. explored this question at some length when they examined the effects of the absence of OPN *in vivo* as well as in rat osteoclasts *in vitro*. Rat osteoclasts that were OPN-deficient displayed low cellular motility and fundamentally less capable of their normal function of bone resorption [117]. Interestingly, these effects were also found to be reversible. Since OPN is known to stimulate CD44 expression on the surface of osteoclasts [134], which is a receptor critical for osteoclast motility and bone resorption, exogenous OPN was added back to the OPN-deficient osteoclasts, upon which osteoclastic

motility was rescued. Not only that, but here again, activation of integrins was observed, most significant of which was the $\alpha_v\beta_3$ integrin, which, as we have already stated, is associated with OPN and CD44 activity. This, in conjunction with Carvalho's study, seems to suggest a bi-directional relationship between integrins and OPN. All told, these *in vitro* results correlated well with their *in vivo* observations of OPN-deficient mice: decreased osteoclast number and function translated to a delayed bone resorption response as well as an increase in bone dimension, as measured in the tibiae. Functionally, the bones of OPN-deficient mice experienced an increase in moment of inertia, which is a measure of structural rigidity and resistance to bending [135]. Obviously, OPN-deficiency produced a very pronounced and distinct phenotype, showing that OPN is necessary for certain bone-maintenance tasks.

Mechanical stress to bone plays a critical role in its own homeostasis. In this guise, OPN-deficiency has also been connected with a decrease in mechanosensitivity of bone cells. Utilizing a similar OPN knockout approach as Chellaiah, Ishijima went one step further to study the mechanotransduction potential of OPN by giving a mechanical signal to bone. By unloading the skeletal frame of OPN-knockout and control mice through tail-suspension, Ishijima discovered that the OPN-knockout mice did not lose bone like the control animals did [136]. One might hypothesize based on Chellaiah's work that this could be because of a decrease in osteoclast quantity and function, thus impairing resorption. Indeed, Ishijima did in fact note that whereas the number of osteoclasts increased in wild-type mice after unloading, the OPN-deficient mice held at a constant number. Even more interesting, measures of osteoblastic bone formation, decreased in wild-type mice, were *also* held constant in OPN-knockouts! Recalling that bone is a dynamic tissue, constantly forming and degrading its own components in response to loading via Wolff's law, this is quite astounding that OPN-deficiency can cause an apparent stagnation of these dynamics, disrupting the osteoblast-osteoclast axis. Similar findings were obtained by Fujihara in another tooth movement system of experiments on mice, who saw a suppression of bone remodelling in OPN-knockout mice as well [137]; even though the tooth movement setup required loading to bone, instead of unloading as performed by Ishijima, the same inability to resorb bone was seen. In addition, Fujihara

also showed that on the genetic level OPN is “hard-wired” to react to mechanical stresses as at least one, possibly more, mechanical stress response element(s) exists in the OPN-promoter region in the 5.5-kb upstream region. So clearly, from the previous discussions, one can at least see that OPN is designed to respond to mechanical force and does so by causing changes in bone in some manner.

More specifically, OPN appears to have sensitivity to both compressive and tensile stresses. Under compressive stress, OPN follows a biphasic pattern of expression in time [138]. Also, stimulation of calcium content in mineralized nodules formed by the osteoblasts was observed. From the links previously established between calcium ion flux and OPN, this is not surprising to find OPN elevation concurrent with calcium elevation. Physiologically, too, this increase in calcium may help in the adaptive response of bone in order to better withstand the compressive force. Concurrently, Morinobu demonstrated in mice that expansive force on bone results in bone formation at its edges, which indicates increased activation and differentiation of osteoblasts at points near application of stress [139]. In his team’s study, OPN levels increased everywhere in the bone specimens where tensile stress had induced a response, whereas conversely, OPN-knockout mice were deficient in bone formation.

1.5 Soluble CD44 (sCD44) Basics

Very little to date is known about *in vivo* functions performed by soluble CD44, or its molecular modulators. Certainly, according to the author’s researches, it has never been illustrated as being a mechanosensitive molecule on its own. On the biochemical level, at least, all human isoforms of the CD44 family of adhesion molecules are encoded by a single gene [140]. Alternate splicing of 12 of the 19 exons in the human CD44 gene leads to the production of multiple variant isoforms [141]. Many CD44 isoforms are tissue specific, but many soluble variant isoform(s) of CD44 (sCD44) has been associated with certain pathological conditions [142]. CD44 exists in several domains at the cellular level (**Table 1.1**), serving various functions at each location. CD44 is also a major receptor for hyaluronan (HA) [117]. It has been proposed that soluble variants of CD44 are either generated through proteolytic cleavage of cell surface CD44 or by de novo synthesis due to alternative splicing. Moreover, some suggest that structural heterogeneity of CD44

isoforms is responsible not only for determining the ligand repertoire of CD44, which includes fibronectin [143], chondroitin sulfate [144], osteopontin [145], and at least two heparin binding growth factors [146, 147], but also for modulating the hyaluronan (HA) binding ability [148, 149]. Hyaluronan (HA), also called hyaluronate or hyaluronic acid, is a glucopolysaccharide widely distributed throughout the body, produced by a variety of cells including fibroblasts and other specialized connective tissue cells [150].

Table 1.1 CD44 forms/domains, mechanisms of generation, and associated functions. Adapted from [140].

Form of CD44	Potential mechanisms of generation	Known and proposed functions
Transmembrane	Synthesized as type-I integral transmembrane protein	1. Cell–cell adhesion 2. Cell–matrix adhesion 3. Signal transduction 4. Pericellular matrix assembly 5. Hyaluronan metabolism
Matrix-associated ectodomain	1. Shedding of transmembrane form 2. Secretion of alternatively spliced form	1. Pericellular matrix assembly 2. Integral component of ECM 3. Agonist/antagonist of transmembrane form
Fluid phase	1. Shedding of transmembrane form 2. Secretion of alternatively spliced form 3. Enzymatic liberation from matrix	Agonist/antagonist of transmembrane- and matrix-associated forms
Intracellular cytoplasmic domain	Presenilin-dependent cleavage of truncated transmembrane form	Regulation of transcription after nuclear translocation

1.6 OPN and sCD44 in Scoliosis

OPN and its receptor ligand, sCD44, were not formerly linked to the AIS disease until the advent of a number of studies led by our own lab. Recently, though, our laboratory concluded a study in which a sizable cohort of severely affected patients, moderately affected patients, asymptomatic “at risk” individuals born from at least one affected parent, and controls was recruited in order to track circulating plasma OPN and sCD44 levels in the blood. We demonstrated elevation of circulating plasma OPN levels on average in affected and asymptomatic persons compared to controls, the latter finding suggesting that these elevations may well precede scoliosis onset [98]. Also, sCD44 levels were significantly lower in severely affected individuals with respect to controls. These findings were further verified using an animal model in C57Bl/6j mice, a strain known to develop scoliosis when maintained in a bipedal state [151]. Neither bipedal transgenic OPN nor sCD44 knockout mice of this strain developed any trace of scoliotic curve, in contrast to their bipedal wild-type counterparts [97]. To our knowledge, this is the only demonstration to date of a relation to AIS from either OPN or sCD44.

1.7 Overview of Mechanotransduction in Humans

One area of research that has not been as extensively followed in regards to AIS, and which could possibly underpin all of the aforementioned hypotheses of its etiopathogenesis, is that of mechanotransduction. Undeniably, the volume of mechanotransduction research is extremely scarce in comparison to other threads of AIS fundamental research that have been followed thus far.

Mechanotransduction is the process by which external mechanical loads or stimuli are converted into biochemical activity [5]. Mechanotransduction in human bone takes place in four distinct phases:

(1) *Mechano-coupling*, the transduction of mechanical force applied to the bone into a local mechanical signal perceived by a sensor cell;
(2) *Biochemical coupling*, the transduction of a local mechanical signal into a biochemical signal and, ultimately, gene expression or protein activation;
(3) *Transmission of signal* from the sensor cell to the effector cell, i.e., the cell that will actually form or remove bone; and
(4) *Effector cell response*, the appropriate tissue-level response.

From the decade of the early 1890s when Wolff's now-classic experiments proved mechanical force as an inducer of bone remodelling effects [152], mechanical loading is now known as a key external signalling stimulus that can drastically alter cellular characteristics and gene expression at a fundamental level. Specifically, the musculoskeletal system responds particularly strongly, frequently utilizing these mechanical signals to direct its adaptive cellular responses to such loads through mechanotransduction. That increases and decreases in these inputs are capable of driving adaptive changes in the skeleton is perhaps most concretely evident when observing that usage-based loading, such as sports and exercise, increase bone density and strength in a site-specific way [153], whereas disuse and unloading such as that experienced by astronauts in weightless conditions or in paralysis results in severe loss of bone tissue [154, 155]. However, the exact cellular mechanisms by which bone adaptation works are poorly understood. This process of bone adaptation requires bone cells to detect

mechanical signals in situ and integrate these signals into appropriate changes in the bone architecture. Likely, the actual mechanosensory process involves the interpretation of a complex amalgam of signals and responses obtained and then combined from structural cellular elements such as ion channels, integrins, connexins, and even other elements of the cell membrane [156].

Mechanical forces are believed to be mediated in cells by so-called "mechanosensitive" genes. These include key mechanosensitive second messenger pathways involving constituitive enzymes (like prostaglandin and nitric oxide synthase) in the cell membrane [5]. This genetic component of mechanosensitivity is perhaps its single greatest determining factor; Robling illustrated this when he saw that differing population densities of osteocytes (supposed "mechanosensor" cells) did not correlate at all with the degree of mechanosensitivity in terms of osteogenesis in response to dynamic mechanical loading in various mouse strains [157].

1.7.1 Key Cellular Mechanotransductive Pathways

Overall, there are five major mechanotransductive signalling pathways identified to date at the cellular level. A map of the interactions among these pathways and the key signaling molecules that they contain which orchestrate the osteoblastic response to mechanical stress are schematically summarized in **Figures 1.7** and **1.8**. Concisely, these are namely:

1. <u>MAPK signalling</u>

The mitogen-activated protein (MAP) kinases (MAPKs) comprise a diverse evolutionarily conserved family of protein serine/threonine kinases that provide a key link between the membrane-bound receptors that receive environmental cues and changes in the pattern of gene expression [158]. Three major groups of distinctly regulated MAPK cascades are known in humans: the extracellular signal-regulated kinase (ERK), the c-Jun NH2-terminal kinase (JNK), and the p38 MAPK cascade [159].

2. Ca^{2+} signalling

Ca^{2+} signalling regulates numerous basic cellular functions, such as muscle contraction, apoptosis, and bone metabolism [160, 161], by ‘‘turning on and off’’ signal transduction pathways and Ca^{2+}-dependent proteins. We have already noted that calcium ion flux is one of the first cellular responses to mechanical stress, and that it is likely linked to cellular OPN expression. Rapid increase in cytoplasmic Ca^{2+} concentrations is also driven by G-protein coupled receptors (GPCRs), molecules well-perceived to be activated as well by mechanical forces [162, 163].

3. β-Catenin/WNT signalling

These WNTs constitute a large family of glycoproteins regulating tissue morphogenesis, cell motility, and proliferation. Although three separate WNT pathways have been identified, the so-called “canonical” β-Catenin/WNT pathway is by far the best well-characterized in the musculoskeletal system. It is a crucial component of nearly every aspect of skeletal physiology, including bone mass accrual, homeostasis, and maintenance [164]. Recent studies indicate that the Wnt/β-catenin signaling pathway has a pivotal role in the ability of bone to sense and consequently respond to alterations of its mechanical environment [165, 166].

4. Integrin signalling

Integrins are cell adhesion trans-membrane molecules serving as receptors for extracellular matrix proteins like collagen and fibronectin [167]. They consist of 18 α and 8 β-subunits, which together form 24 distinct αβ-heterodimers, depending on cell type and function [168]. Osteoblasts express a wide range of integrins, including αv, α1, α2, α3, α5, α6, α8, and β1, β3, β5 [169]. Integrins have been implicated in an incredible selection of stress-related phenomena in several musculoskeletal cell types including fibroblasts, myoblasts, and endothelial cells [170, 171].

5. Nitric Oxide and Prostaglandin signalling

These two molecules are often expressed in concert when stimulated. Nitric oxide is a very reactive molecule, reacting very quickly with oxygen, and has emerged as a mediator in many physiological processes including vascular relaxation and neurotransmission [172]. Nitric oxide also affects both the osteoblastic and the osteoclastic lineages of bone

cells. Specifically, low nitric oxide levels induce osteoblast growth and differentiation, as well as osteoclast function, while high levels arrest these activities and promote apoptosis [173, 174]. Prostaglandins are powerful anabolic bone factors promoting bone formation under mechanical stimulation [138, 175]. These anabolic effects of prostaglandins *in vivo* are associated with the increased induction of transforming growth factor-β (TGFβ) by RUNX2, a gene known to possess matrix formation capacity [176].

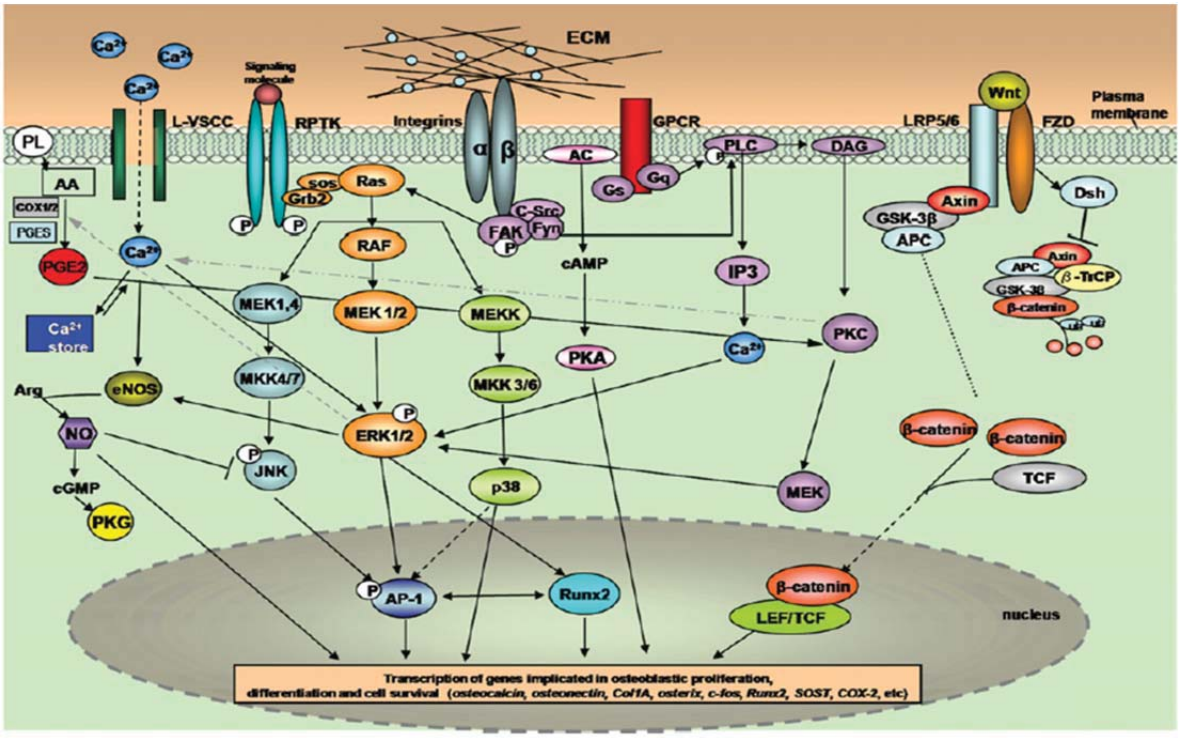

Figure 1.7 Interaction map between key mechanotransductive pathways. Adapted from [159].

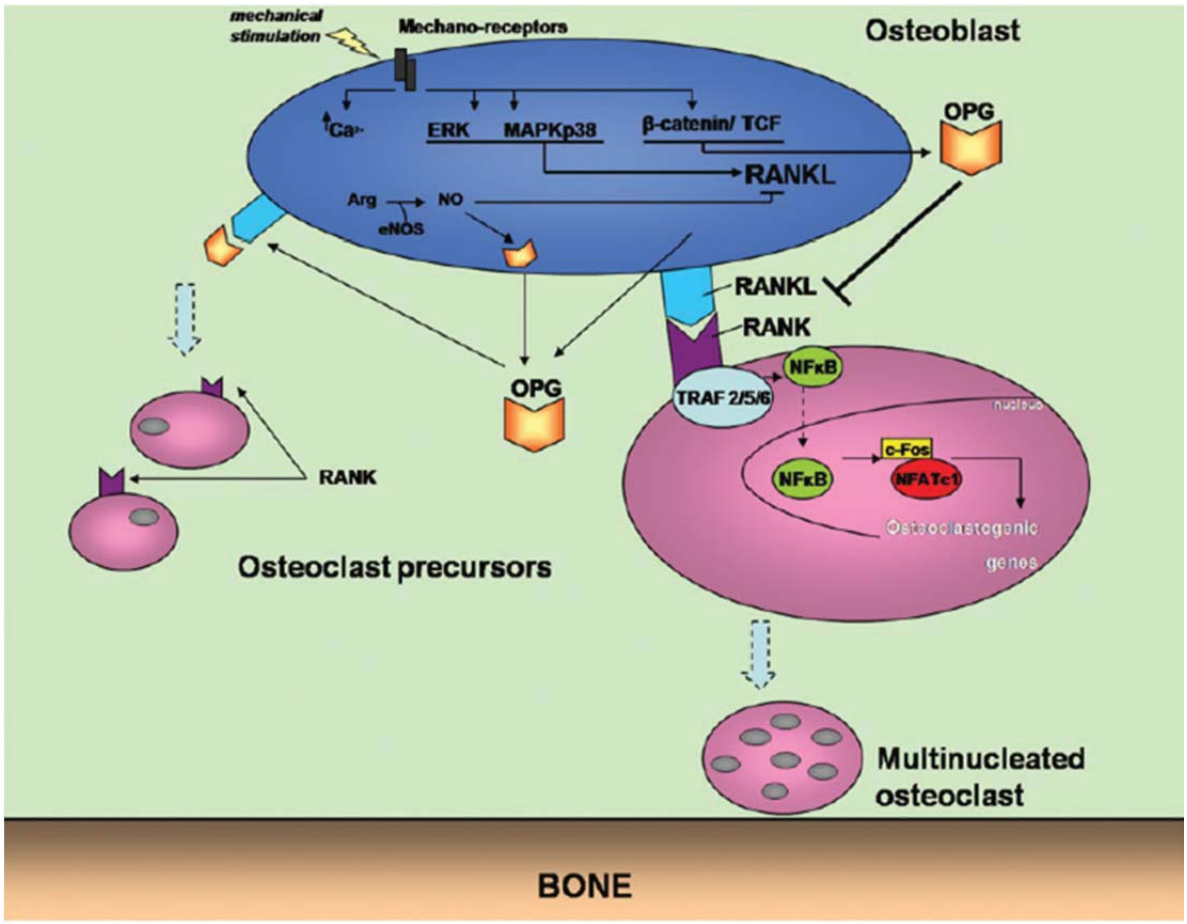

Figure 1.8 Schematic representation of basic molecular events underlying the impact of mechanical stimulation on osteoblastic response. Adapted from [159].

1.8 Biomechanical forces/Mechanotransduction known in AIS

The purpose of the skeleton is to provide mechanical support and protection for vital soft tissues such as the brain and cardiovascular system, and to create the system of articulated levers that comprise the locomotive system that functions to move us around. Its structural success is a function of its mechanical properties – its stiffness, resistance to fatigue, and its resilience [156]. Components of the musculoskeletal system in general have the remarkable ability to optimize their morphology in response to their functional environment. In scoliosis, the characterization of this altered morphology is of great interest as it may provide clues to its etiopathogenesis.

Biomechanical forces have long been believed to play a sizable role in AIS etiopathogenesis. As it pertains to our theme, mechanical forces and stimuli have been hypothesized as a key agent altering the morphology of the spine and inducing progression of scoliotic curves numerous times; most recently, for example, by Stokes

with his so-called “vicious cycle” hypothesis [177], where mechanical stimuli are clearly labelled as factors abetting the progression according to the Hueter-Volkmann effect [178] (eccentric pressure changes direction of spinal growth). Essentially, according to this idea, vertebral body wedging characteristic of scoliosis results from asymmetric muscular loading; once a scoliotic curvature to the side exists in a spine, the weight of the body segments superior to that curve in upright positions of the trunk creates a lateral bending moment that tends to increase the vertebral wedging, and thus curve severity, in a self-perpetuating manner.

Now, it is well-established that a scoliotic spine and its surrounding musculature experience a very different stress and loading pattern than does a normal spine, for instance. Indeed, most biomechanical research in AIS has been directed towards exposing these loading differences between normal and scoliotic spines. Formerly, particularly in the 1970s and 1980s, factors such as spine slenderness/flexibility [179, 180] and strength or myoelectric activity in trunk muscles [181, 182] were championed as possible relevant factors in AIS progression, before ultimately being refuted and abandoned as implausible [183]. Today, modern thinking tends to revolve around two issues: asymmetries and buckling load theory.

By asymmetries, one refers to any type of disproportion in shape, growth, or loading of the spine as a factor in the disease. Certainly AIS is a disease characterized by some sort of planar imbalance [184]; however, the exact nature of these inequalities is hotly debated. For instance, there are those who maintain that AIS is best characterized by a left-right asymmetry in the lateral plane [185], while others argue just as fervently that scoliosis is not at all a left-right problem, but rather one dominated by front-back asymmetries, according to the evidence [186]. Regardless, it is certainly a very current topic in AIS biomechanical research, even if some simulations *in silico* have indicated that no asymmetry need be present at all for curve induction [187]. The second issue, buckling load theory, is driven by the resemblance of a scoliotic spine to a structural column that has, in a manner of speaking, exceeded design specifications, and failed in a way similar to how a metal beam might well do under too great of a bending moment about its axis.

According to this concept, the scoliotic spine has given way due to exceeding the critical load given by Euler's formula for bending moment:

$$P_{cr} = \frac{N \times EI}{L^2}$$

where N = constant allowing for end conditions, E = Young's modulus, I = moment of inertia, and L = effective length of column. Again, there is fantastic debate in this area, but no conclusive proof of this so-called "Euler theory" has ever been given [185].

The key features of note that I would like to draw out regarding the themes of all these researches enumerated to date is first that their focus has been exceedingly localized, almost entirely in and around the spinal region, completely marginalizing the possible modulatory effects that other forces present elsewhere in/on the body may be having. Moreover, as there exist many practical and ethical difficulties in being able to elaborate such mechanisms at the human level directly *in vivo* [186], many of the hypotheses dealing with how components of the human musculoskeletal system sense and/or respond to biomechanical loads in AIS and in general have hitherto relied mostly on *in vitro* methods, inferential animal models, or computational *in silico* methods [188-190], representing a great limitation in the existing literature today.

1.9 Static vs Dynamic Loading in the Musculoskeletal System

The simplest types of physical stimuli are tensile (stretching) and compressive stresses (although in reality, tensile loading is a very rare occurrence in nature). These occur with mechanical loads applied perpendicular to the cross-sectional load-bearing area, as opposed to shear stresses which are incurred when the application of force is parallel with the cross-sectional area. All of these kinds of stresses can be further subdivided into static and dynamic groupings. However, static and dynamic loading are virtually never occurring mutually exclusively *in vivo*.

In static loading, the amount of applied force is temporally constant; loading of the skeleton due to gravity (weight of the individual) is the simplest example of this kind of loading. In real physiological organisms, too, gravity is usually the only significant kind of naturally-occurring static loading taking place [191]. Throughout the musculoskeletal

system, this mode of loading is generally not conducive to bone anabolism, but rather resorption. For instance, Cancel et al. revealed in 2009 decreases in production of certain collagens (type II and X) in the extracellular matrix due to static loading, causing concurrent reductions in growth plate thickness and bone formation [192], possibly due to hindrance of cellular hypertrophy [193, 194]; these histomorphological effects would be consistent with the results of numerous other studies [195-199]. Lee further added credence in 2007 to this idea of static loading being an inducer of resorption, verifying that several powerful osteoclast activators, most notably interleukin-6 (IL-6) and alkaline phosphatase (ALP) were significantly up-regulated in periodontal ligament cells in response to static compressive loads [200].

In contrast, dynamic loading is variable with time. Experimentally, the kinds of dynamic loadings utilized or simulated are periodic waveforms in time (usually sinusoidal or trapezoidal for ease of analysis [191]). All manner of harmonic frequencies in complex waveforms are found with dynamic loading *in vivo* with the musculoskeletal system. Studies of human gait have revealed spectral frequencies as high as 75 Hz existing internally [201]. Systemic vibration stresses from causes such as inherent system noise or even certain types of external bone therapy approaches can be of frequencies from 10-100 Hz [202-204]. However, bone remodeling has been determined to favour frequencies of loading between 5-10 Hz [205].

On the whole, dynamic loads seem to be more stimulating to cells and tissues than static ones [204, 206-210], particularly the low-magnitude, high-frequency variety. As we shall see later, modern researchers generally concur that this is attributable to the fact that fluid flows in the musculoskeletal system are principal drivers of bone remodelling, and these flows can only exist under dynamic loading conditions [5, 191]. In 1998, Turner further elaborated that the adaptation process is not dependent on loading that is routine, but rather is “error-driven”, by which he meant that only those dynamic loads that were abnormal, unusual, or out-of-the-ordinary drew cellular responses because they were not used to them [211]. Certainly in terms of bone remodeling effects, it is the only type to which bone responds in an osteogenic fashion. Also, versus those engendered by static loading, detrimental effects to bone histomorphometry from dynamic loads are

minimal [212]. Finally, dynamic loading is of key importance in determining peak bone mass [213].

1.10 Mechanosensing in the Musculoskeletal System

There is ample evidence to suggest that most cells in the body are in fact able to sense their mechanical environment. At the present level of understanding, there are some generalities common to nearly all types of cells responding to mechanical force. For instance, MAPK expression is activated by tensile, compressive, and shear forces in cells of the endothelium, smooth muscles, and bone [159]. Logically, the most prominent cells in the musculoskeletal system, the osteoblasts, osteocytes, even chondrocytes, should have the same basic machinery responding to physical stimuli. Detectable differential responses will then arise out of the site- and time-specific genetic expression patterns in the cells in question.

The ability of bone to react to mechanical stimuli has been repeatedly demonstrated already [157, 204, 205, 214, 215]. Moreover, mechanical responses of the osteoprogenitor family of cells, which includes stromal cells, osteoblasts, and osteocytes, have also all been well documented [216-219]. However, it is often difficult to identify a sole critical responding cell type. By and large, though, the osteocyte is generally the most cited as having the primary function of responding to mechanical stimuli, though for the sake of completeness, we must note that myoblasts, osteoclasts, as well as several other types of musculoskeletal cells all have evidence of responsiveness to force as well [136, 203, 220, 221]. We give now a brief treatment of mechanotransduction in the two most significant and commonly studied cell types.

1.10.1 Component Cells: Osteocytes

Descending from osteoblastic progenitor cells, osteocytes are the most numerous type of musculoskeletal cell in bone. The idea that osteocytes could function as mechanosensors for the musculoskeletal system had been conjectured for many decades. Intuitively this makes sense as one would imagine that components best positioned to detect force in any system (in this case, bone) would be those dispersed throughout it. Yet the first hard evidence of their role in this way was remarkably only published in 1989,

when Skerry et al. showed activation of osteocytes in turkeys via increased glucose-6 phosphate dehydrogenase activity following loading mimicking the flapping of wings [222]. Since then, this responsiveness has been confirmed numerous times with different markers [223, 224], and by 2002, there was general consensus of osteocytic mechanosensitivity and ability to convert mechanical strains to biochemical activity [131, 217, 218]. Dentin matrix protein 1 (DMP1), a matrix protein expressed in jaw osteocytes, has been shown to increase expression after application of force to the jaw simulating the movement of teeth such as would be expected under the effect of corrective dental appliances [225]. What has not been agreed upon, however, includes the nature of mechanical strains to which osteocytes are sensitive and the form of the biochemical signals, and most importantly, whether it is the major responder to mechanical strains. It has been suggested that osteocytes are more sensitive to shear than short-term loading by Klein-Nulend in 1995 [130], but there is great conflict upon this subject [226].

1.10.2 Component Cells: Osteoblasts

The osteoblast class of cells, as well as chondrocytes can also be activated or altered by force. In a functional sense, osteoblasts can be thought of as the effector of the response to be carried out as instructed/informed from the osteocytes, and are thus of equal importance to bone responses. As the direct precursor cell type to osteocytes in the differentiation line [227], one might expect quite a degree of similarity between parent and daughter cell type. However, given the relatively small number and location pattern of osteoblasts on the surface in bone as compared to osteocytes, it is not likely to be the cell type to dominate mechanical response, since these disadvantages would require the osteoblast to be extraordinarily sensitive to be capable of sensing mechanical stress [218, 228]. However, what is possible is that as the parent cell to the more likely candidate as mechanosensor, osteoblasts could indirectly influence the mechanosensory ability of the body via its own differentiation pathways; that is, perhaps the efficiency and quality of this ability to transform into osteocytes may be a means of affecting mechanosensitive capacity *in vivo*.

1.11 Fluid Flows in the Musculoskeletal System

Fluid flows moving over cells are physiologically encountered in the musculoskeletal system and are thus scientifically relevant. In vivo, the bone fluid existent from the region of the bone vasculature through the canaliculi to the lacunae of the surrounding mineralized tissue normally experiences a heterogeneous pressurization and depressurization due to deformation of the mineralized matrix from normal mechanical loading and unloading via locomotion of the skeletal frame [131, 229-232]. Although this loading/unloading cycle is not usually sinusoidal, it is repetitive, leading to cycles of forward and reverse physiological fluid flow following pressure gradients. A representative diagram of this occurrence is shown below in **Figure 1.9**.

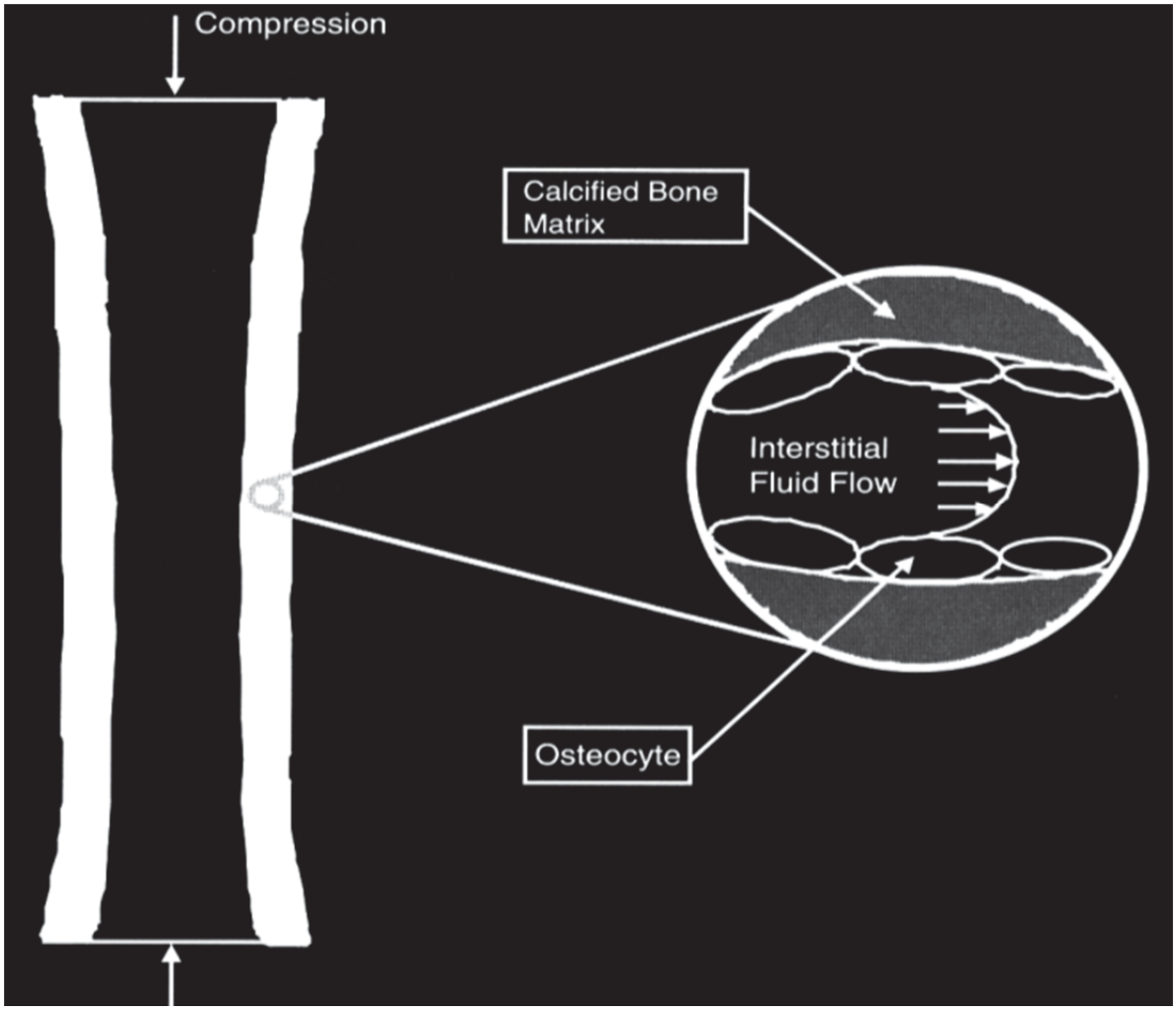

Figure 1.9 Representative diagram of fluid flow in bone. Loading/unloading cycles cause localized pressure gradients, inducing fluid flow.

Fluid flow over cells may seem fairly innocuous at first glance, but it may in fact have profound implications in bone remodelling through mechanotransduction. In vitro, it has been shown to be a great stimulator of bone cells [165, 233-235]. It may surprise some to learn that it has even been shown to be a more significant effector of biochemical activity in musculoskeletal cells than the more widely known conventional modes of mechanical strain [231, 234, 236]. Yet, despite all this, it remains a wholly unexplored region of study with respect to AIS, with absolutely no presently existing literature on the matter.

The mechanics through which fluid flow is connected to cellular deformation is quite complex to study. Nevertheless, You et al. in 2001 considered osteocytes and created a mathematical model predicting load-induced fluid flow can lead to shear forces on the plasma membrane of the cellular process and drag forces on the fibrils in the pericellular matrix [128]. Oddly enough, You's model also indicated enhanced tissue strain at the osteocytic cellular level because of the structural organization of the cells, lending credence to the notion that osteocytes are important mechanosensitive cells. Now, Jacobs has suggested that if the idea is correct that fluid flow induces bone remodeling, then dynamic loading should also induce it, since the dynamic loading will create movement of fluid [237]. Conversely, static loading should not cause bone remodeling due to the lack of fluidic movement created. In fact, this was already documented by Liskova and Hert in 1971 in rabbits [238, 239], later confirmed by Lanyon and Rubin in 1984 through their avian ulna modeling technique [206].

1.11.1 Fluid Shear Stress Study Using Parallel Plate Flow Chambers

Study of fluid-induced shear stress is typically performed on an *in vitro* basis, with the aid of designed devices known as parallel plate flow chambers (PPFCs). These devices allow researchers to examine responses of biological cells to theoretically constant and uniform wall shear stresses. **Panel A** of **Figure 1.10** illustrates an older schematic design of a classic PPFC, designed to work with rectangular microscope slides, while in **Panel B**, a representative drawing of a newer kind to accept standard cell culture dishes is shown. In either case, the principles of operation are the same. Briefly, the parts of the apparatus are held together by vacuum pressure (suction) applied to a vacuum ring around the

exterior of the region of cells to which fluid flow will be applied. Because of the rubber gasket that separates the lower part containing the cells under study from the upper securing piece (usually fabricated in Plexiglas), a long and extremely thin rectangular chamber is created between them, into which fluid can be infused at desired flow rates and patterns in order to shear the cells adherent at the bottom of the chamber [240, 241]. Under assumption of laminar flow conditions and use of Newtonian fluids, the fluid velocity profile inside the chamber follows the Hagen-Poiseuille relation [242], and thus the applied shear stress to cells will be relatively constant and uniform over the vast majority of the flow field, represented by the relation derived from the famous Navier-Stokes equations of fluid mechanics [243],

$$\tau = \frac{6\mu Q}{\beta h^2},$$

where Q is the flow rate, τ is the shear stress, μ is the dynamic viscosity, β is the chamber width, and h the chamber height. One notes the convenience of this method of study, as the resultant shear stress is a function only of fluid and chamber geometry parameters.

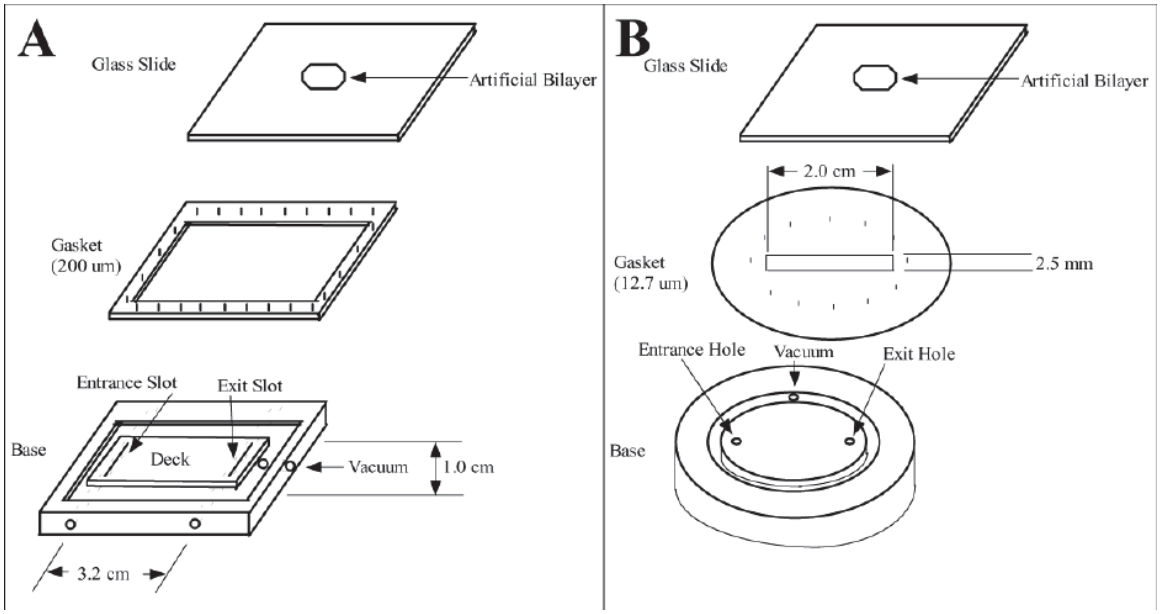

Figure 1.10 Schematic of older, rectangular design of PPFC (**Panel A**), and of newer circular design for use with cell culture dishes (**Panel B**). Adapted from [240].

1.11.2 Fluid Flow Schemes

There have generally been three types of fluid flow studied experimentally: steady [244], pulsatile [245], and oscillatory [246, 247]. Example flow rate profiles can be found later in **Figure 4.1**. We now pass under consideration each of these flow regimes in turn and summarize their most important characteristics and known features.

1.11.3 Steady Fluid Flow (SFF)

One observes from the referenced diagram (**Figure 4.1**) that steady flow is analogous to static loading discussed previously, as the flow rate of fluid over cells is a constant. This mode of fluid flow is quite unrealistic in true physiological settings, as it is difficult to conceive of any situation in the human body, let alone the musculoskeletal system, whereby matter or fluid is simply in infinite supply to be in flux unidirectionally over a set of cells for extended periods of time. Rather, this mode of flow really only exists in the literature due to its simplicity in experimental execution. It was useful as a first-attempt in the early proof-of-concept studies of fluid flow using PPFCs, but has since mostly fallen out of fashion. Nevertheless, in the literature, steady flow does exist, though it has been consistently been demonstrated that its effects are generally of lower magnitude than more dynamic flows. Under steady flow, it has been shown that bone cells undergo slow and steady viscoelastic creep in response [214]. It is capable of stimulating to a limited extent nitric oxide production [248], an important effector of osteoblast and osteoclast activity. Lastly, steady shear stress augments proliferation and differentiation of human osteoblasts [249], though at a slower rate than the other types of flows [250, 251].

1.11.4 Pulsatile Fluid Flow (PFF)

In contrast, pulsatile flow is akin to an on/off switch controlling flow, where there is either a certain flow rate in one direction or no flow at all. Since, by definition, a unidirectional pulsatile flow implies mass transport from one region to another, not to mention the existence of a source and a reservoir, pulsatile flows are likely not the most physiologically relevant in the musculoskeletal system, as bone is largely a “closed” system; i.e. it is not simple for matter to enter and leave. Pulsatile flows do exist in physiological systems of the human body, most notably in the circulatory system, causing

hemodynamic shear stresses which have been studied a number of times [245, 252-254], where of course there is an obvious source and reservoir (heart). This is not to say that PFF has been completely ignored; research has shown that PFF induces many similar effects to oscillatory flows. It increases cellular prostaglandin E_2 production just as oscillatory flows do [255]; however, these changes are not long-lasting in general [216], whereas oscillatory flow-induced changes tend to endure.

1.11.5 Oscillatory Fluid Flow (OFF)

Finally, oscillatory fluid flow (OFF) is best described as a sinusoidal pattern of flow, with equal forward and reverse flow rates occurring at a given frequency over the period of oscillating flow. It is dynamic and most akin to the oscillatory description of flow in nature, making OFF *in vitro* the most similar to real musculoskeletal physiological conditions. When bone is exposed to mechanical loading fluid in the matrix is pressurized and tends to flow into Haversian canals. As loading is removed (e.g. during the gait cycle) the pressure gradients, and consequently the direction of fluid flow, are reversed resulting in a flow-time history experienced by the cells that is oscillatory in nature [128]. Under this flow regime, cells behave primarily as elastic bodies just as their function dictates they should [244].

OFF has received increasing attention from the scientific community in the last 10 years since the aforementioned 1998 Jacobs study, which turned out to be something of a landmark in mechanotransduction research. Previous to Jacobs, only steady or pulsatile flow profiles had been considered. But in 1998 Jacobs experimented with OFF in vitro in bone cells (osteoblast-like cells) for the first time, and he compared the magnitude of effects on bone cell mechanotransduction caused by these three flow regimes. His group's results clearly showed OFF to be significantly less stimulatory, in general, than pulsatile or steady flow in terms of the response magnitude and fraction of responsive cells. As well, the dynamic regimes' ability to stimulate decreased with increased flow frequency. Both intuitively and physiologically, these results actually make sense and complement each other. For if OFF is indeed the flow regime one would normally encounter in the body most of the time, one would not expect systems or cells in the body to have increased responsiveness to it, since, as noted before, physiological systems tend to adapt in a

minimalist manner such that they only give significant differential responses to stimuli and phenomena that are *abnormal* [211]. This could also explain the second major finding as well, then, since the human skeleton's dynamic loading is normally mostly the low-magnitude, high-frequency variety [202, 203]. The declining frequency-response may serve as an adaptive mechanism to counteract the increased shear stress associated with increasing frequency predicted by the theoretical model of Weinbaum in 1994 [233], but this has not been confirmed to date. The musculoskeletal system may also employ this frequency response as a defense against negative bone remodelling. Kim et al. in 2006 conducted experiments in bone marrow stromal cells (BMSCs) whereby the continuum between receptor activator of nuclear factor kappa B ligand (RANKL) and osteoprotegerin (OPG) levels (whose relative levels dictate osteoclastic formation and differentiation) under the influence of OFF was shifted in favour of osteoclastogenesis inhibition through upregulation of OPG and corresponding downregulation of RANKL [247]. In sum, with respect to scoliosis mechanotransductive signalling, it is conceivable that a direct change or introduction of an element that may induce a change in fluid flow regime or pattern may have a part in the stimulatory changes present in AIS.

As an interesting side note, Jacobs himself also noted the peculiarity that a greater response associated with pulsatile and steady flow corresponds to the two flow regimes in which cells are exposed to net fluid transport as opposed to the one that does not (OFF), despite the fact that the peak shear stresses induced by all three regimes were the same. This is indicative of a chemotransport-dependent mechanism, perhaps the chemotransport of a "mystery" serum factor (factors?) that may play a large role bone cells' response to fluid flow [131, 232, 256, 257]. Such a hypothesis would agree with published results for bone cells by Allen in 1997 [258], when he demonstrated that sensitivity of bone cells to fluid flow is dramatically increased with the addition of serum to the media. What could this factor, or these factors, be? There may be a number, but there is one possibility that could have great implications to scoliosis if its levels or localization were altered. Jacobs' experimental approach towards measuring cellular responsiveness by differentially quantifying intracellular Ca2+ concentrations ([Ca2+]), a well-defined method of measuring cell responsiveness, could suggest a factor with a

relationship to [Ca2+] levels, which, based on our earlier discussions, we have seen that osteopontin [OPN] strongly possesses. Taken together, all this hints at some fascinating possibilities within the domain of fluid shear stress study.

Chapter 2 : Project Rationale

The literature review of the preceding chapter permits us to extract the following most salient of points from it:

- AIS is the most common spinal deformity occurring in childhood, affecting millions of adolescents worldwide and costing healthcare systems billions of dollars each year.
- The precise etiological foundation of the AIS disease remains unknown despite a multitude of effort and diverse approaches through the years.
- An important universally acknowledged factor in AIS development and progression is that of biomechanical force. However, heretofore, most focus has been on forces and their alterations in and around the spinal region. Our own informal observations indicate that this narrow viewpoint may be slightly short-sighted; mechanical forces elsewhere in the body may also be contributing.
- Mechanical force, particularly dynamic mechanical force, has the capacity to drastically alter cellular characteristics and gene expression at a fundamental level in many different cell types. This process is called mechanotransduction.
- Mechanotransduction is a relatively new and emerging angle of research in the field of AIS study that holds many possibilities for novel insights.
- Lacking, however, in the domain of AIS and biomechanical force study in general, is scientific evidence in human tissues and cells, at either the *in vitro* or *in vivo* level.
- *In vivo*, our lab has demonstrated a novel correlation between circulating levels of OPN and sCD44 and AIS severity in human patients.
- OPN itself is a highly mechanosensitive molecule, as has been demonstrated numerous times. sCD44 however, has not previously been associated with mechanical response.
- *In vitro*, fluid-flow induced shear stress is a powerful effector of mechanotransductive response, even more so than the more standard and commonly considered mechanical strain.

- OFF is the most physiologically relevant mode of fluid shear stress in the musculoskeletal system.
- Overall, fluid shear stress study has been completely neglected thus far in AIS mechanobiological research, with no prior literature in the matter.
- Using PPFCs is an elegant, convenient way to study fluid shear stress in desired cell types of choice in humans, leading to superior levels of evidence and potentially new insights in AIS mechanotransduction with little in the way of ethical or practical difficulties as has been a large obstacle to advancement in this domain up to now.

Analysis of these considerations leads to the following hypothesis:

“AIS patients possess important distinguishing characteristics in the way they respond to mechanical force, not exclusive to the spinal region, at both the cellular level (in terms of gene expression) as well as globally at the *in vivo* level (in terms of the scoliosis marker OPN and its receptor sCD44).”

As well, using these now-established precepts as guidelines will allow the formation of an experimental design permitting new characterizations of AIS mechanotransduction in humans at the *in vivo* and the *in vitro* level.

Two principal objectives will guide the formulation of the experimental method of this exploratory project that will help aid in verifying the research hypotheses:

1) Characterize, at the *in vivo* level in humans, any mechanotransductive relationship between bodily-applied mechanical force and the molecules OPN and sCD44 across differing classes of patients.
2) Observe, for the first time, the broad-spectrum differential gene expressional effects of the most physiological relevant mode of fluid shear stress (OFF) in AIS patients compared to unaffected individuals on an *in vitro* basis, with the help of PPFCs.

The realization of these two objectives will be presented in the next two chapters, Chapters 3 and 4, respectively, through the presentation of separate scientific manuscripts

for each. These articles briefly present the methodological aspects and justification of chosen parameters, where applicable, before proceeding to an elaboration of the *in vivo* OPN and sCD44 differences in mechanical force response, and then to a first comparative look at mechanotransductive differences in AIS-affected individuals. Following that, Chapter 5 will provide a complement to the methodological description of certain protocols used in the scientific articles, furnished where more detail may prove helpful for other researchers wishing to follow this vein. A general discussion, where recommendations for future directions are included, and then the conclusion complete this document.

Chapter 3 : *In vivo* Assessment of Mechanotransduction in AIS using osteopontin (OPN): Towards an Early Detection Test?

For the manuscript below, the first author conceived the experimental design of this *in vivo* work, helped in the recruitment of subjects, and supervised the execution of the experimental protocol for each patient tested. Also, he analyzed the resultant experimental data and wrote the manuscript. The contribution of the first author is therefore evaluated at 85%, for this manuscript to be submitted to *The FASEB Journal* (*FASEBJ*).

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The institutional review board of The Sainte-Justine University Hospital approved the study.

3.1 Abstract

Background and Objective: Adolescent idiopathic scoliosis (AIS) is the most commonly occurring musculoskeletal deformity among children today. Previously, we demonstrated that the level of plasma osteopontin (a mechanosensitive molecule) and sCD44 (a known OPN binding partner) in the body is a strong indicator of this disease's progression and severity, and that these changes are observable before scoliosis onset. We now examine in this clinical exploratory pilot study the global mechanotransductive properties of these markers at the *in vivo* level.

Methods and Materials: To date, 38 test subjects have been recruited between the ages of 9-17, each of whom fall into one of four subject groups: i) surgical cases (pre-surgery, Cobb angle > 45°) (n=9), ii) moderately affected cases (Cobb angle 10-44°) (n=13), iii) controls (n=10), or iv) asymptomatic children at risk of developing scoliosis matched for age and gender against healthy controls (n=6). An initial blood sample was taken from each subject to establish a baseline value of circulating OPN and sCD44 in plasma. One of the arms from each subject was then wrapped with an inflatable cuff which applied a dynamic, pulsatile, compressive pressure of variable amplitude from 0-4 psi at 0.006 Hz to the arm for a period of 90 minutes. At intervals of 30 minutes after the start of force application, additional blood samples were taken in order to monitor circulating plasma OPN and sCD44 levels in subjects.

Results & Discussion: Our results from this small cohort of test subjects indicate that average circulating OPN levels of all four experimental groups increased over the course of the 90 minutes of mechanical stimulation. Interestingly, there was a trend found, that patient grouping and ΔOPN were strongly significantly correlated (one-way ANOVA $p=0.003441$), with average group ΔOPN declining as the group curve severity increased. No correlation was found between patient grouping and ΔsCD44, however (one-way ANOVA $p=0.542$).

Conclusion: This study developed a useful method to discriminate between patient *strata* using the behaviour of OPN levels in response to mechanical loading. We quantifiably characterized the differences in normal subjects and AIS patients of varying severities in

terms of the circulating OPN response, demonstrating a significant separation among severity groups in this regard. The findings obtained seem a promising first step to future use of OPN and its provoked response to mechanical force as a means of predicting the risk of developing scoliosis among asymptomatic children and of spinal deformity progression.

3.2 Introduction

Adolescent Idiopathic Scoliosis is a disease of the spine that affects a significant proportion (1-3%) of young adolescents (by definition, between the ages of 10 years and 17 years, 11 months). Although much studied, the etiopathogenesis of the disease remains unclear. What's worse, there does not exist a proven procedure at present that is capable of identifying those in the population at risk of developing scoliosis or of its progression in pre-existing cases. Consequently, the application of current treatments, such as bracing or surgical correction, is delayed until a significant deformity is detected or until a significant progression is clearly demonstrated, resulting in a delayed and less than optimal treatment. Therefore, there is a great need for innovative clinical tests to identify asymptomatic children at risk of developing scoliosis as well as those who are symptomatic in order to predict who may be at risk of scoliotic curve progression. Ideally, in our view, such a test would be cheap and simple, as well as offer an answer to the diagnostic question rapidly. As well, in consideration of the fact that such a diagnostic would be aimed towards a pediatric population, one would prefer that the test also be as non-invasive and non-threatening as possible.

Previously in our laboratory, we have demonstrated a strong association of increased circulating osteopontin (OPN) levels and concomitant decreased levels of sCD44 (a known decoy receptor with OPN binding capabilities) with the risk of induction/progression of a scoliotic curve [97, 98]. Osteopontin is a phosphorylated glycoprotein expressed in a wide array of bodily tissues and cell types [112], and is typically involved in numerous inflammatory and defensive mechanisms in the body [115]. Indeed, it is clear from the literature that elevated OPN levels can be triggered by many etiological causes involving different genes and environmental factors. As well, at least on an *in vitro* level, OPN expression has also shown mechanotransductive sensitivity [259, 260]. Lacking, however, has been corresponding evidence *in vivo* in humans, and indeed mechanotransduction study in general, due to the obvious practical and ethical obstacles to overcome in many cases [186]; thus, most effort in this vein has been aimed towards indirect inferential studies using animal models [119, 137, 138]. This is most unfortunate, especially for those studying the fundamental basis of scoliosis, as biomechanical forces

are generally acknowledged as a probable key factor in AIS development [188, 261, 262], so knowledge of its possible mechanotransductive effects in humans would be invaluable to our basic understanding of the condition.

Based on some informal circumspect observations in our own laboratory, though, consideration of the effects of mechanical force on all possible components of the musculoskeletal system, rather than focusing solely on the spine, may be necessary. Take for instance the example of children undergoing dental realignment through the use of braces to the teeth (but otherwise completely normal); they will experience the application of mechanical force to the jaw, a part of the body which has absolutely no anatomical relation to anything in the spinal system. Yet, our as yet unpublished observations of some children who present to the dental clinics at CHU Sainte-Justine indicate that these children tend to have a significantly higher incidence of scoliosis compared to normal populations, roughly 3-5 times higher, in fact. This correlates with an earlier European study demonstrating much the same phenomenon [6].

Given all this, we wished to test the following hypotheses in our study: a) by supplying an externally applied mechanical stimulation to the body, circulating levels of OPN will be stimulated *in vivo* in all patients, reminiscent of previous *in vitro* results; b) that there exists a global and generalized difference in mechanotransduction observable in terms of OPN and/or sCD44 between controls and scoliotic patients, not restricted to merely the spinal region. If proven, we believe that these principles may be exploitable in the framework of developing a first practical, clinical scoliosis test, based on observation of differential responses of OPN in response to bodily-applied mechanical forces.

3.3 Methods and Materials

3.3.1 Study Population

The Institutional Review Board of CHU Sainte-Justine approved this study. Parents or legal guardians of all participants gave their informed written consent, and minors their assent. Subjects were recruited from among the general patient population of the orthopaedic clinic of CHU Sainte-Justine. Four particular classes of patients aged 9-17 were sought out for the purposes of this study: i) controls; ii) asymptomatic subjects; iii)

moderately affected (Cobb angle 10-44°); and iv) severely affected individuals (Cobb angle ≥ 45°). A person was deemed to be affected if history and physical examination were consistent with the diagnosis of idiopathic scoliosis and a minimum of a ten degree curvature in the coronal plane with vertebral rotation was found on radiograph. The Cobb angle as measured on the radiograph then determined a patient's status as either moderately or severely affected. Each subject was examined by an orthopaedic surgeon using Adam's forward bending-test with a scoliometer. Asymptomatic at-risk children, defined as those with less than a 10 degree curvature but with a family history of AIS, were recruited and examined in our special early screening clinic at CHU Sainte-Justine, where such children are known to us and present routinely in order to maintain a close watch on the status of their spine, given their increased risk of scoliosis. Controls did not have any family history of AIS or any spinal curvature greater than 10 degrees. These subjects were drawn from among those who either a) had already participated in one or more previous studies carried out by our laboratory, or b) presented to our orthopaedic clinic at CHU Sainte-Justine by chance, for reasons other than scoliosis. In all cases, family history of AIS was established by asking subjects and their accompanying relatives about the presence of a spinal deformity affecting a family member. Subject exclusion criteria from data analysis included: i) regular utilization of contraceptive drugs; ii) BMI greater than 35; iii) employment of any external physical apparatus to help stabilize the spinal cord. Patient demographic and clinical data are presented in **Table 3.1**.

3.3.2 Mechanical Force Stimulation

Upon arrival, participants in the study were asked to lie flat on their backs on a hospital bed and advised to keep still as much as possible during the subsequent protocol. After allowing the patient to settle and rest on the bed for 5-10 minutes, an initial blood sample was drawn from one of the arms of the patient. Subsequently, a pair of rectangular medium-sized air bladders from an ABR Therapeutic Air Massager device (Panacis Medical, Ottawa, Ontario) were arranged and attached to the other arm above the elbow, in much the same manner as one would a sphygmomanometer, as described in the product documentation. The rectangular bladders were oriented such that their lengthwise direction ran parallel to the length of the patient's arm, and were maintained

firmly in place using the Velcro belt provided with the machine. This ABR device has been certified by numerous health and regulatory agencies in North America, the EU, and around the world, including a Health Canada authorization for clinical use in supplying pneumatic compression on the human body for therapeutic ends to patients. The massager device was reprogrammed from the manufacturer's preset settings in order to produce cycles of inflation/deflation of the bladders at a frequency of approximately 0.006 Hz, supplying a pulsatile compressive stress ranging from 0-4 psi to the area of the arm covered by the medium-sized air bladders. Patients experienced the stimulus for a total of 90 minutes, during which time blood samples from the non-stimulated arm were taken, every thirty minutes, making a total of four blood samples (roughly 5-6 ml each) drawn per patient, including the initial at t=0 min. Subjects were strictly confined to the bed for the duration of the experiment, to allow for uninterrupted periods of mechanical stimulation.

3.3.3 OPN and sCD44 Enzyme-linked Immunosorbent Assays

Blood samples from IS patients, asymptomatic at-risk children and healthy control subjects were obtained in order to determine plasma levels of OPN and sCD44. These were collected in EDTA-treated tubes and then centrifuged. Derived plasma samples were aliquoted and kept frozen at -80°C until thawed and analyzed. Plasma concentrations of OPN and sCD44std (standard isoform) were measured by capture enzyme-linked immunosorbent assays (ELISA) according to protocols provided by the manufacturer (IBL, Hamburg, Germany). The OPN ELISA kit measures total concentration of both phosphorylated and non-phosphorylated forms of OPN in plasma whereas the sCD44std ELISA kit detects all circulating CD44 isoforms. All ELISA tests were performed in duplicate and the optical density was measured at 450 nm using an AsysHiTech Expert-96 microplate reader (Biochrom, Cambridge, UK).

3.3.4 Patient Environmental Factors

During the 90 minutes of mechanical stimulation, a part of the time was also effectively utilized to pose a series of questions to participants and their accompanying parent(s)/guardian(s), aimed at determining the presence of possible environmental and/or lifestyle factors that might have affected results. Questions asked over the course

of the study are listed in **Table 3.2**. Responses were recorded, tabulated, and classified into groups, where necessary.

3.3.5 Statistical Analysis

Average group levels of OPN and sCD44 are presented as mean ± SD. Statistical significance p-values of differences in group levels of OPN and sCD44 between control, asymptomatic, moderately affected, and severely affected patients was respectively assessed in the first instance using linear regression models with a one-way ANOVA. The effects of age and gender were then individually studied, each in combination with grouping, using a two-way ANOVA with weighted means and Type I sums of squares to account for unbalanced sample sizes (i.e. age and group, followed by gender and group as factors in the analyses), where age groups for ANOVA analyses were defined as younger subjects between 9-12 years of age and those between 13-17 years. Patient environmental factors were compared across experimental groups with Fisher's exact test for discrete variables and again *post-hoc* for any factors identified as significant, and a one-way ANOVA for continuous variables (average age in each group). P-values < 0.05 were considered statistically significant. The software used for all statistical computations was R, version 2.13.1 [263].

3.4 Results

3.4.1 Study Population

We investigated four experimental groups: severely affected, moderately affected, Between January 2010 and March 2011, a total of 38 subjects (mean age 13.69 ± 2.25) of various ethnicities were recruited into this study. Breaking down this cohort, we had 9 severely affected (mean age 14.26 ± 1.27), 13 moderately affected (mean age 13.43 ± 2.50), 6 asymptomatics (mean age 13.16 ± 2.78), and 10 control subjects (mean age 13.87 ± 2.41), according to our definitions set forth previously. Patient and control subject demographic and clinical data are summarized in **Table 3.1**.

3.4.2 Circulating OPN and sCD44 Levels

Initial starting values of circulating plasma OPN levels in blood were not found to be significantly different between experimental groups (one-way ANOVA $p=0.20$), as shown in **Figure 3.1**. Average circulating plasma OPN levels of all four experimental groups

increased over the course of the 90 minutes of mechanical stimulation. A raw box plot of subject ΔOPN (i.e. $OPN_{t=90\ min} - OPN_{initial}$) by experimental group is shown in **Figure 3.2**. Interestingly, there was a trend found, that patient grouping and ΔOPN were strongly significantly correlated (one-way ANOVA p=0.0034), with average group ΔOPN declining as the group curve severity increased. Tukey's HSD post-hoc test showed that there was very statistically significant variation between the severely affected group and the control (p=0.0029), but not between other pairwise group combinations, though there was suggestive borderline significant correlation suggested between moderately and severely affected groups (p=0.084) as well as between the control and asymptomatic groups (p=0.0593). No statistically significant correlation between sCD44 levels and group severity was found (one-way ANOVA p=0.542), as shown in **Figure 3.3**.

3.4.3 Effects of Age and Gender

To study whether ΔOPN was affected by the age and sex of subjects, we carried out two-way ANOVA analyses with unbalanced sample sizes and Type I sums of squares, first with gender and experimental group as factors. Using this model construct, it was found that gender had a statistically significant effect on ΔOPN, in conjunction with experimental group (gender p = 0.0047, experimental group p = 0.0027, with gender as the first factor), with the female subgroup of each severity class generally displaying a somewhat lower average ΔOPN value in comparison to their respective male subgroup, except for the 10-44° group, where the internal male and female subgroups had virtually identical average ΔOPN responses. We then analyzed the data with the factor order reversed, and found that gender still had a statistically significant effect on ΔOPN, in conjunction with experimental group (gender p = 0.022, experimental group p = 0.00098, with experimental group as the first factor). A quite statistically significant interaction was found between gender and experimental group (interaction p= 0.0285). By contrast, age grouping was statistically significant, in conjunction with experimental group, when age group was considered as the first factor (age group p = 0.0286, experimental group p = 0.0064), but only borderline statistically significant when considered as the second (age group p = 0.052, experimental group p = 0.0044), with no significant interaction between the two factors (interaction p= 0.793). Here, the younger age subgroup (ages 9-12) in each

severity class exhibited a somewhat higher average change in OPN levels in response to the mechanical stimulation than their respective older age subgroup (ages 13-17), although in the most severely affected 45° group the two age subgroups were nearly at parity with one another.

3.4.4 Patient Environmental Factors

No subjects reported any regular consumption of alcohol or tobacco. Experimental groups did not differ significantly in most demographic or lifestyle factors that may have impacted scoliosis status, modulation of OPN expression, and/or typical biomechanical loading patterns in a subject's body over the course of a reasonable timeframe in a patient's history prior to the day of testing, as displayed in **Table 3.3**. There was, however, a very significant group difference found in terms of AIS prevalence in patient family histories ($p = 3.37 \times 10^{-5}$). Post hoc Fisher exact test analysis of distributions between combinations of two groups revealed these variations lay chiefly in comparisons with the control group ($p=7.15 \times 10^{-3}$, 3.65×10^{-3}, 1.25×10^{-3} for controls vs. severely affected, moderately affected, and asymptomatics, respectively) but not others. Interesting to note, though not meeting our overall p-value criterion, was borderline significance found in terms of the age group factor ($p = 0.098$) as well as the frequent consumption of poultry/eggs ($p = 0.052$).

3.5 Discussion

This exploratory pilot study seminally examined general mechanotransduction, not restricted to merely the spinal region, in scoliosis patients in terms of the OPN marker at the *in vivo* level. In this cause, we recruited a small cohort of subjects with varying scoliosis severities to test global response of circulating plasma OPN levels in the blood to a dynamic mechanical stimulus applied externally to the arm, a site far from the spinal area. From our results, we showed that a) OPN was indeed universally responsive, on average, in all four experimental subgroups, as per our test results illustrating OPN increases across the board in **Figure 3.2**; b) there was a distinct and statistically significant relationship between group severity and ΔOPN response but not ΔsCD44; and c) this relationship was also significantly dependent on both age group and gender, particularly gender, wherein females and/or older patients demonstrated lower ΔOPN responses on

average. In addition, one observes a relative homogeneity within each experimental group in terms of the ΔOPN response, as evidenced by the reasonable standard deviations in each, and the absence of any particularly gross outliers. This seems to suggest a degree of robustness in terms of the induced OPN response with respect to the presence of a number of environmental factors that might otherwise have an effect on OPN itself and/or the regular biomechanical loading condition of the subject. These form significant findings in our opinion, as to our knowledge, this is the first time such a mechanotransduction-linked OPN responsiveness has been established at all at the global *in vivo* level in humans. Further, these results from stimulation support our initial notion that non-spinal forces on the musculoskeletal framework can also have important mechanotransductive effects, not to be neglected in study of scoliosis patients.

Prior to this study, most work on OPN and indeed mechanotransduction overall was usually inferential in nature and/or performed *in vitro*, usually in animals [139, 264] or in cultured cell lines [259, 265]. Such prior studies confirmed OPN to be a mechanosensitive molecule, increasing its expression in tissues and cells under study significantly after mechanical stimulation. Our own *in vivo* findings here are reminiscent of those results on balance, yet there are some interesting features of note. First off, although all subjects' circulating OPN level did respond positively to the stimulation, the pattern of correlation was not what we might have expected. That is to say, in the present case, patients in the AIS-affected groups responded less forcefully than controls. Given that biomechanics and mechanotransduction are thought to have a major role in AIS, and that mechanical force is a known inducer of OPN whose elevated expression we have previously associated with development and progression of AIS [97], we initially believed that mechanical forces/loading may act through the OPN pathway to produce AIS phenotypes. If that was true, one might have expected a certain parallel here, but this was clearly not the case, as the opposite effect was observed. Since the initial OPN levels before stimulation were not significantly different between groups as per **Figure 3.1**, the explanation of already-elevated OPN levels making further stimulation of production more difficult in affected individuals would seem unlikely. However, it might simply be due to the difference between the two methods whereby here we employed an active dynamic

stimulation to try and provoke an OPN response, whereas the previous study monitored the passive circulating OPN expression (no stimulation whatsoever) in subjects over time points within a period of 18 months. Thus, we may simply be observing a different behaviour of OPN under a different testing and measurement schema. Whatever the origins, this is surely a phenomenon that merits further study.

The data in **Figure 3.1** showing no significant difference in initial starting OPN levels between groups in and of itself might appear a slight contradiction to our earlier findings pertaining to OPN and scoliotic development, where mean passive OPN levels were found elevated on average among more severe cases. One possible explanation is the difference in which the blood was drawn from patients: in the earlier study, patients were generally upright in a sitting position. Since we desired to create a scenario where, as far as possible, the only loading on the musculoskeletal system would be our own applied stimuli, we requested all patients to lie flat on their backs and as still as possible for the entire duration of the test, including all blood sampling. It is conceivable to speculate that the removal of most loading on the musculoskeletal frame as would happen when lying down helped to equalize initial subject OPN values, especially when one considers that the major difference between patient groups was the degree of spinal curvature, and presumably by implication, any variations in OPN that may have been connected to differing spinal loading patterns. Removing this causative factor thus may remove the previously observed effect in the first study. If true, we arrive thus at the key supposition that both spinal and non-spinal loading are both capable of effecting mechanotransductive responses, measurable at least in terms of the OPN marker.

In terms of patient environmental factors and demographic data gathered, these were tracked in order to check any potential biasing factors that may have affected results or trends. Apart from the more obvious ones pertaining to smoking and alcohol, family history of AIS, as well as medications taken regularly by patients, the others were followed as the impact of their presence in a lifestyle has, in a peripheral manner in some cases, been shown to be associated with higher incidences/risks of developing scoliosis. Specifically, questions 5-8 in the questionnaire (**Table 3.2**) were designed to explore possible differences in responses to mechanical force in subjects who practice certain

types of physical activities/sports, musical instruments, and/or follow different diets. For instance, there has been evidence of increased incidence of scoliosis in ballet dancers [31, 32] gymnasts [33], and musicians (especially females) [35, 36]. Also, the role of estrogen, particularly in concert with melatonin, has been recently a hot topic in studies of possible mechanisms of AIS etiopathogenesis [27, 266]; thus, foods like eggs or chicken that are extremely high in estrogens consumed as a regular staple of one's diet is a possible factor we wished to learn more about. Lastly, physical activities that require spending time outdoors (in sunny conditions, presumably) as well as certain foods are capable of elevating levels of Vitamin D in the body, a substance with possible modulatory effects on OPN itself [125], as well as calcium ion flux [126], an important precursory event to OPN expression *in vitro* in response to mechanical stress [128].

Indeed, a statistically significant difference was noted in the cohort as to the presence of a family history of AIS, mostly in connection with comparisons involving the control group. However, this is probably most easily explained by the necessarily imposed requirements of certain classes of subjects, like controls (absence of any AIS in family history) and asymptomatic patients (must have family history of AIS) as well, whereas the two AIS groups held no such restrictions; hence, this finding was most likely unavoidable. There were also two borderline significant environmental factors found, age group and level of poultry/egg consumption. As to the former, a slight bias in age group participation is to be expected, particularly in the severe surgical group. Young patients with curves severe enough to even warrant consideration for surgery are simply rarer to find; indeed, in many clinical studies, the age at surgical intervention on average seems to fall well above the 9-12 year old age range [94, 267-269]. Regardless of the origins of this disparity in the cohort, this feature should probably be kept in mind when interpreting our two-way ANOVA analysis of effect of age group on OPN response. Differences in the frequency of consumption of foods rich in estrogenic content are more difficult to explain and indeed fertile ground for speculation; we can find no reason why such a variation should exist. Given estrogen's implication with scoliosis as well as mechanotransduction in general (at least at the *in vitro* level [270, 271]), perhaps this may in fact represent a confounding factor in the results from some groups; unfortunately, there is no way of telling at present.

Some limitations and issues in this study must be acknowledged. First, we admit freely that 38 subjects is a relatively small cohort, surely not powered enough for hard conclusions from a statistical standpoint. To be sure, given the exploratory pilot nature of this study, we did not compute formal sample size. Certainly then, effects and observations, though promising, should be interpreted with some caution. Second, unbalanced group sizes in comparison, though statistically and computationally still viable thanks to certain specialized techniques, are still cause for concern at certain points in the analysis. In particular, to take one example, in comparing age grouping, the >45° group contained only one subject aged 9-12 against eight in the older 13-17 year block. Clearly this degree of imbalance is problematic in the understanding of such factors' role in this study. Happily though, both issues will be easily resolvable in any more definitive and better powered future studies, simply by recruiting sufficient numbers of subjects. As well, the analysis methodology developed here would be valid and easily scalable to such a future study.

Going forward, if speaking from a purely basic science standpoint, it would be interesting to test OPN response under this schema with varying sites of stimulus application, durations, and/or magnitudes of pressure to observe any sensitivity to these aspects. Clinically, however, is where the applicability of these results may have the most impact. The notable lack of quick and simple diagnostic tools has hampered clinicians dealing with scoliosis cases, delaying treatment in many cases and resulting in less than optimal patient outcomes. Generally, a period of 12-18 months is presently required before making prognosis judgements of patients [98]. A practical diagnostic test requires an ability to identify high-risk subjects in a quick and simple manner, with a methodology easy to adapt into a medical setting. The method outlined in this study has certainly suggested promise in differentiating AIS severity classes, in terms of the OPN marker. Additionally, if developed and proven conclusively, we believe it would offer a quick, simple, and medically convenient way of testing for scoliosis in 90 minutes for high-throughput patient screenings in hospitals, schools, and clinics. To realize this possibility as we envision it, we would require a larger, sufficiently powered study. This will serve to not only to properly assess and evaluate effects, but also to establish baseline ΔOPN

response ranges for each severity group, against which a potential patient's test result would be compared for classification purposes. It will also enable one to analyze the long-term predictive power of the test results. One would have to track patient trajectories over several years of those who undergo testing, in order to see if certain patterns emerge, i.e. develop a diagnostic framework. For example, if an asymptomatic patient comes back with a test result falling more in the range of a severe scoliotic patient as per our scheme, we would need to know if that result was at all an accurate depiction of the patient outcome later on. All in all, it is some distance away, but nevertheless an exciting opportunity to explore.

3.6 Conclusions

In summary, this study was, we believe, a seminal one directed towards finding useful ways to discriminate between patient *strata* using the behaviour of OPN levels in response to mechanical loading. We addressed a novel mode of stimulating OPN expression for study for the first time at the *in vivo* level in humans, as well as characterized (quantifiably) the differences in normal subjects and AIS patients of varying severities in terms of the circulating OPN response, demonstrating a significant separation among severity groups in this regard. Taken together, the returns from this study could benefit scores of people worldwide, forming an important first step to the eventual development of diagnostic tools to identify in a single assay of 90 minutes asymptomatic children at risk of developing scoliosis rather than 12-18 months, and possibly even to assess the risk of curve progression at an early stage in those already affected.

3.7 Acknowledgements

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Table 3.1. Subject demographic and clinical data.

#	Experimental Group	Age at Tlme of Testlng	Gender	AIS Family History	Curve Type	Cobb Angle
1	>45°	12,6	F	Yes	lTrTlL	30-71-34
2	>45°	13,8	F	Yes	lTL	59
3	>45°	13,6	F	Yes	rTlL	47-50
4	>45°	13,6	F	Yes	rTlL	57-44
5	>45°	13,8	F	Yes	lTL	54
6	>45°	13,7	F	No	lTrTlL	32-51-24
7	>45°	15,3	F	Yes	rTlTL	29-53
8	>45°	15	F	No	lTrTlTL	14-41-52
9	>45°	16,9	M	Unknown	rTlTL	39-59
10	10-44°	9,5	F	Unknown	rT	11
11	10-44°	10,9	F	Yes	rTlL	36-40
12	10-44°	12	F	Yes	rT	16
13	10-44°	11,7	F	Yes	lTrT	23-25
14	10-44°	11	F	No	lTL	11
15	10-44°	11,6	M	Yes	rTlL	18-14
16	10-44°	13,7	F	Yes	lTL	16
17	10-44°	13,8	F	Yes	rTlTL	43-24
18	10-44°	14,7	F	Unknown	lTL	16
19	10-44°	16,3	F	Yes	rTlL	16-16
20	10-44°	16,3	M	Yes	rL	57
21	10-44°	16,2	M	No	rTL	35
22	10-44°	17	M	No	rTlL	21-30
23	Asymptomatic	9,4	M	Yes	lT	5
24	Asymptomatic	10,8	F	Yes	rTlL	5-6'
25	Asymptomatic	11,5	M	Yes	N/A	0
26	Asymptomatic	12,8	F	Yes	rTlTL	8-8.
27	Asymptomatic	15,2	F	Yes	rTlL	4-4,
28	Asymptomatic	15,4	M	Yes	rL	6
29	Asymptomatic	17	F	Yes	N/A	N/A
30	CTRL	10,8	F	No	N/A	N/A
31	CTRL	12,8	M	No	N/A	N/A
32	CTRL	13	F	No	N/A	N/A
33	CTRL	15	F	No	N/A	N/A
34	CTRL	15,7	M	No	N/A	N/A
35	CTRL	15,9	M	No	N/A	N/A
36	CTRL	15,5	M	No	N/A	N/A
37	CTRL	16	F	No	N/A	N/A
38	CTRL	9	M	No	N/A	N/A
39	CTRL	15	M	No	N/A	N/A

Curve type code: r = right; l = left; T = thoracic; L = lumbar; TL = thoracolumbar; N/A = not available. 9 severely affected (mean age 14.26 ± 1.27), 13 moderately affected (mean age 13.43 ± 2.50), 6 asymptomatics (mean age 13.16 ± 2.78), and 10 control subjects (mean age 13.87 ± 2.41).

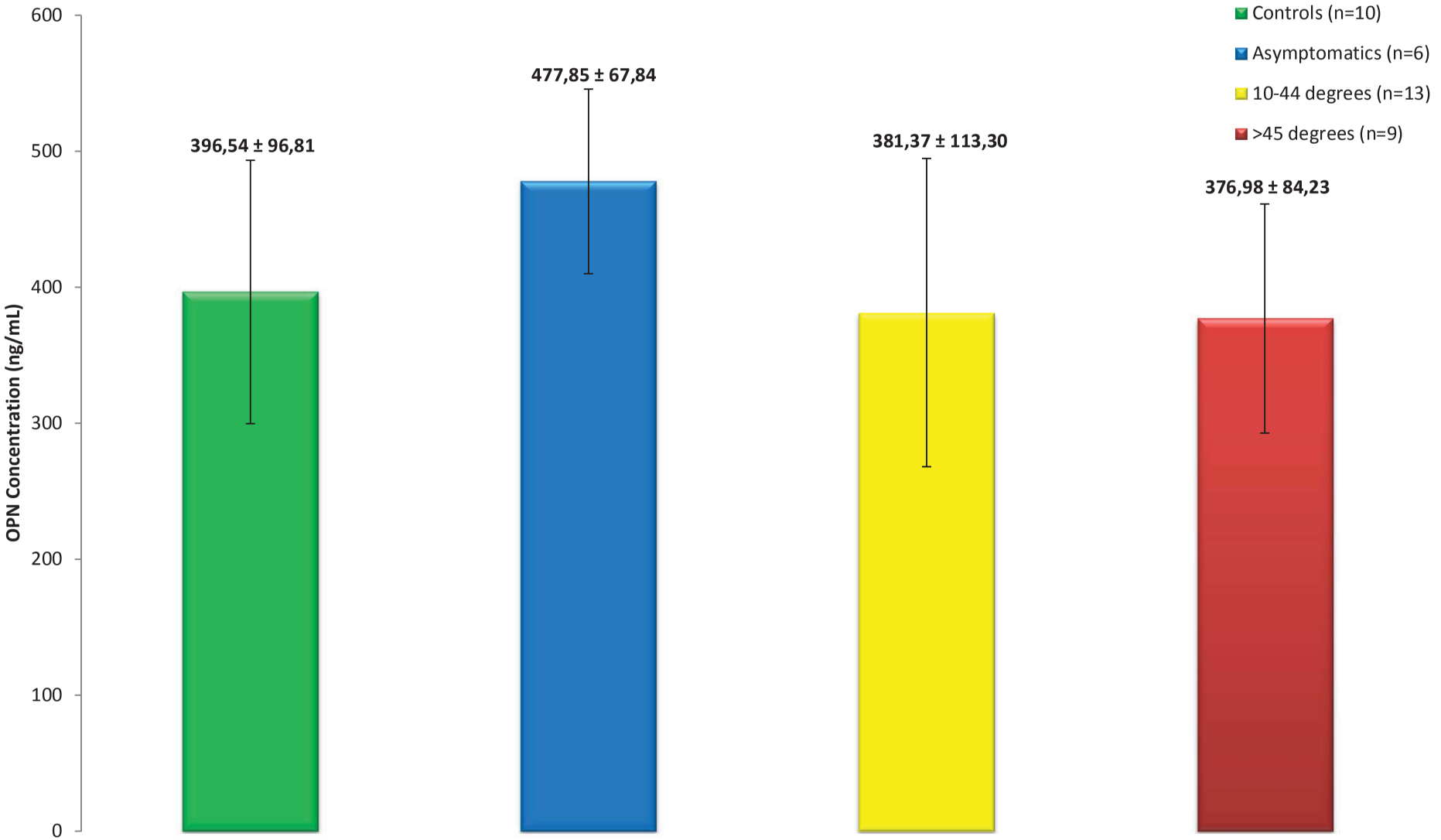

Figure 3.1. Average initial circulating OPN levels (mean ± SD) among experimental subgroups prior to mechanical stimulation. No significant difference was found between groups (p=0.20, one-way ANOVA).

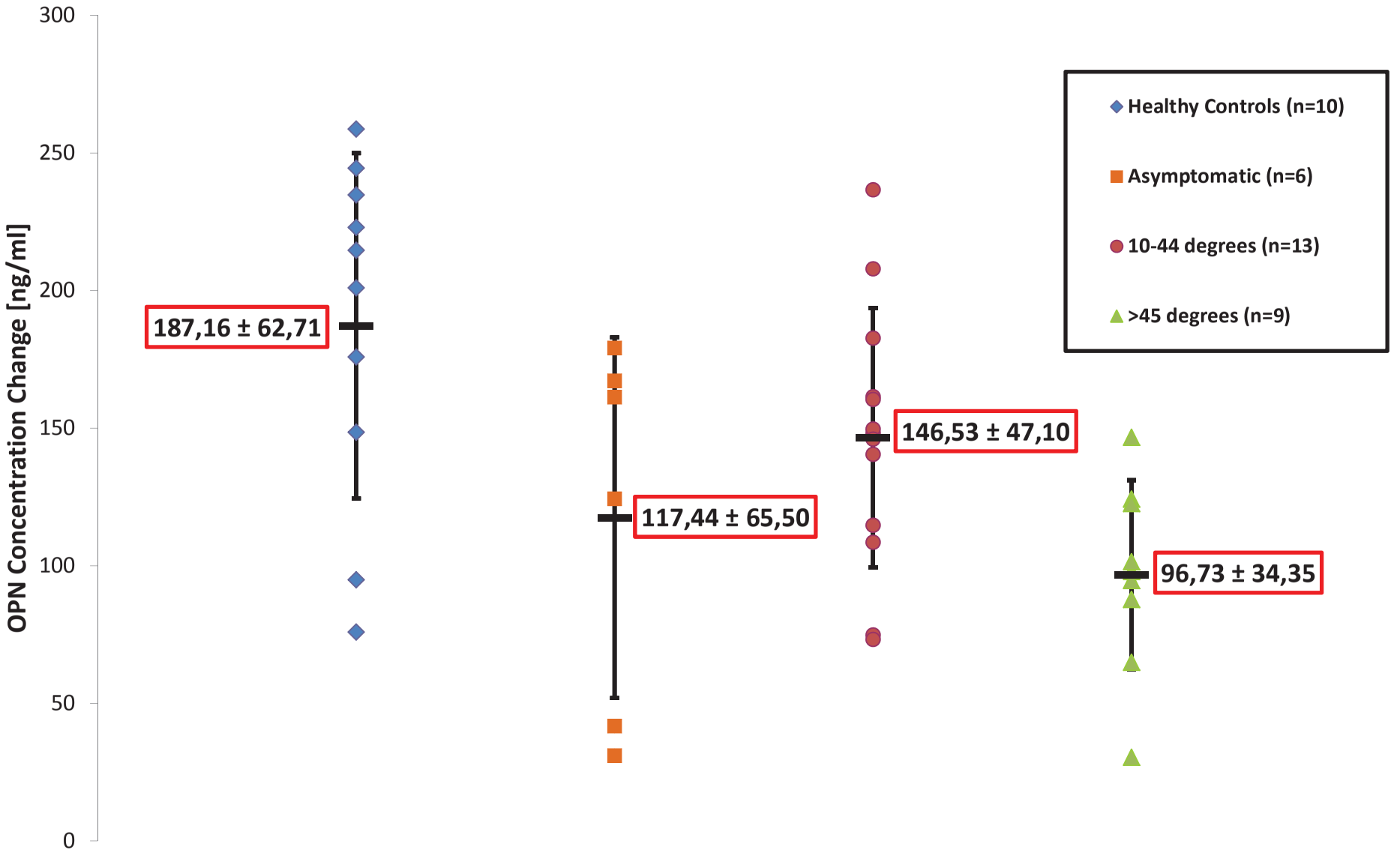

Figure 3.2. Average change in OPN levels (mean ± SD) among experimental subgroups after 90 minutes of periodic compressive mechanical stimulation at 0.006 Hz, varying from 0-4 psi. A strongly significant difference was found between groups (p=0.0034, one-way ANOVA).

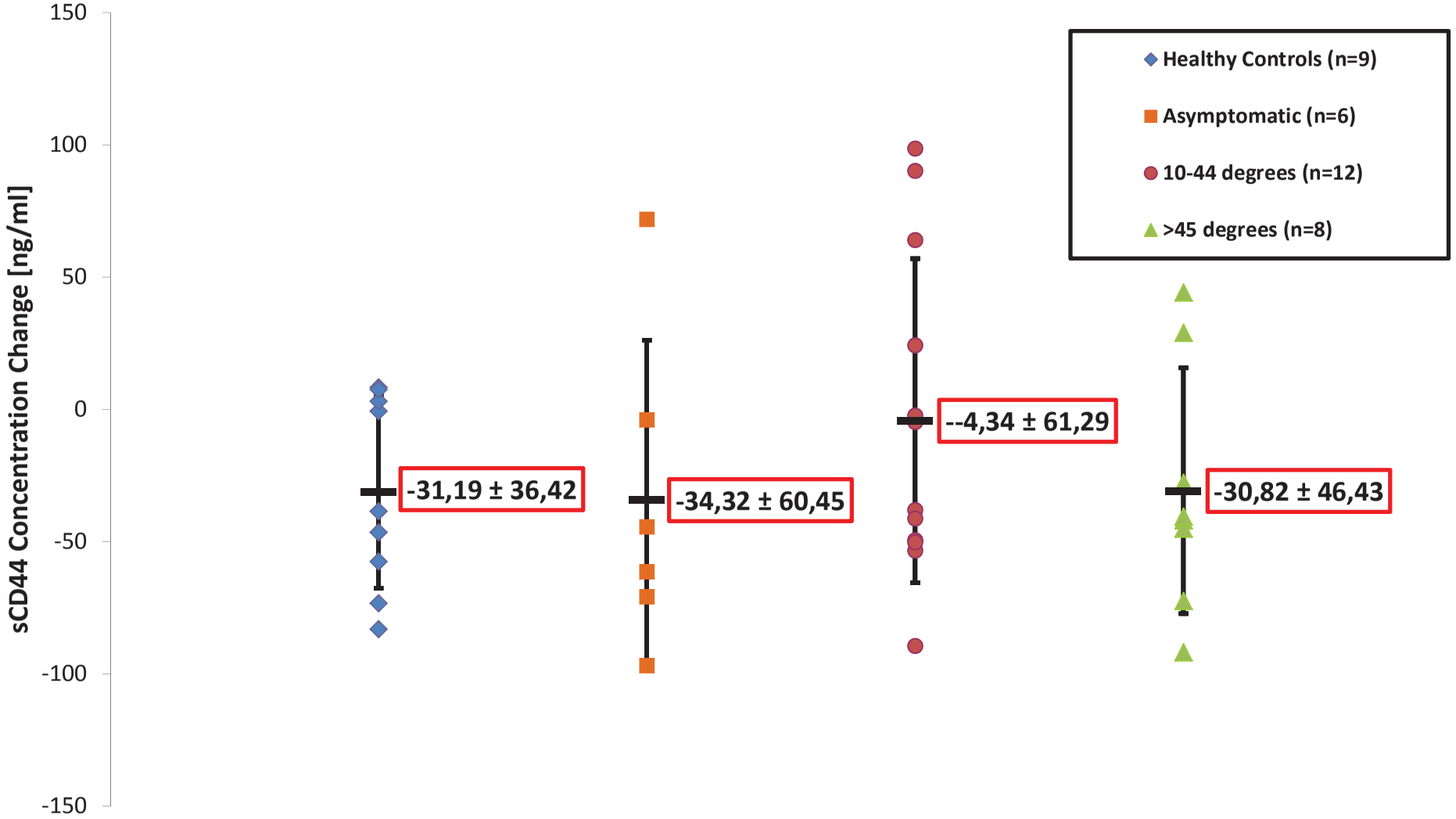

Figure 3.3. Average change in sCD44 levels (mean ± SD) among experimental subgroups after 90 minutes of periodic compressive mechanical stimulation at 0.006 Hz, varying from 0-4 psi. No significant difference was found between groups (p=0.542, one-way ANOVA).

Question
1. Do you smoke?
2. Do you drink alcohol?
3. Do you have a family history of AIS?
4. What medications, if any, do you take regularly?
5. Are you physically active? What sports, if any, do you participate in on a regular basis? How often do you participate, and for how long per week?
6. Do you play a musical instrument? If so, how often do you practice?
7. Did you eat before arrival? If so, what?
8. Do you consume eggs and/or poultry on more that 5 days out of 7 in a week?

Table 3.2. Environmental factors questionnaire as posed to all subjects participating in study.

	>45°	10-44°	Asymptomatics	Controls		P-Value
Age (mean ± SD)	14,26 ± 1,272	13,44 ± 2,50	13,16 ± 2,784	13,87 ± 2,41		0,771
9-12 years	1	8	3	3		*0,098*
13-17 years	8	5	3	7		
Gender (n)						
Female	8	9	3	4		0,135
Male	1	4	3	6		
Family History of AIS (n)						
Yes	5	9	6	0		**3,37E-05**
No	2	3	0	10		
Unknown	2	1	0	0		
Medications (n)						
Antihistamines	0	0	2	0		0,101
Psychopharmacological	1	1	0	0		
Bronchodilators	1	1	1	0		
None	7	11	3	10		
Physical Activity (n)						
<2 hrs/wk (low)	4	3	1	3		0,838
2-9 hrs/wk (med)	5	8	4	5		
>10 hrs/wk (high)	0	2	1	2		
Musical Instruments (n)						
Yes, frequently (>10 hrs/wk)	0	1	0	0		0,61
Yes, regularly (2-9 hrs/wk)	2	2	1	0		
No/sporadically (<2 hrs/wk)	7	10	5	10		
Meal eaten before test (n)						
Yes	7	11	6	7		0,596
No	2	2	0	3		
Consumption of Eggs/Chicken on 5 or more days per week (n)						
Yes	3	0	1	4		*0,052*
No	6	13	5	6		

Table 3.3. Environmental factor and demographic data of study participants. Statistically significant p-values as per significance criterion are in bold in last column (AIS family history); borderline significant factor p-values italicised (Age group, Consumption of Eggs/Chicken on 5 or more days per week). P-values calculated by Fisher exact test; for the statistically significant factor of AIS family history, post-hoc Fisher exact tests were computed on distributions between every possible group combination. Here, only combinations involving comparisons with the control group post-hoc were found significantly different ($p = 7.15 \times 10^{-3}$, 3.65×10^{-3}, 1.25×10^{-3} for controls vs. severely affected, moderately affected, and asymptomatics, respectively).

Chapter 4 : A New Angle of Mechanotransduction in Idiopathic Scoliosis: Osteoblast Response to Fluid Shear Stress

For the manuscript below, the first author a) conceived the experimental design of this *in vitro* study performed in osteoblasts sampled from severely affected AIS patients as well as controls; b) performed the cell cultures, RNA extractions, and subsequent qRT-PCR experiments; c) calculated the experimental mechanical and fluid parameters. As well, he analyzed all resulting experimental data (including those obtained from microarray experiments) and wrote the manuscript. The first author's contribution is thus evaluated at 95%, to this work intended for submission to *The Journal of Bone and Mineral Research* (*JBMR*).

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The institutional review board of The Sainte-Justine University Hospital approved the study.

4.1 Abstract

Introduction: It is generally well accepted in scoliosis research that mechanical forces, especially the internal biomechanical forces of the musculoskeletal system, could well have a major role in the induction and pathogenesis of the disease through possible aberrant mechanotransductive responses. However, as evidenced by the dearth of literature on the subject, a largely neglected area of exploration remains the effect of native physiological fluid shear stresses caused by fluid movement over musculoskeletal cells. These fluid shear stresses are omnipresent and universal in all humans, regardless of age, gender, fitness level, etc., which means that studying it could very well go a long way towards establishing a more rational basis of understanding fundamental differences as to mechanotransduction in scoliosis patients as opposed to normal cases. This study, thus, is aimed towards satisfying these ends.

Objective: Examine differences in osteoblastic gene expression, in scoliosis vs. non-scoliotic patients under typical physiological conditions of fluid shear stress.

Methods and Materials: Osteoblasts from 12 subjects (3 controls, and 3 AIS patients for each of the 3 functional scoliosis subgroups as determined by the classification system developed by Moreau et al., herein referred to as Group I, II, and/or III patients), were cultured for use with our parallel plate flow chamber (PPFC) apparatus setup, which permits application of fluid shear stress patterns to cells in a predictable, controllable

manner. All subjects were females between 11-17 years of age exhibiting a double major curve. Twenty-four hours before starting shear stress experiments, cells were starved with 2% FBS medium. After starvation, the subcultured cells were placed into our PPFC setup, where a dynamic, sinusoidal and oscillatory fluid shear stress pattern was applied at 2 Pa, 0.5 Hz for 90 minutes. No-flow controls were established for all patients as well. Immediately following the 90 minute period, cellular RNA was isolated and harvested using the TRIzol technique. Overall gene expression changes across RNA samples were measured using the Illumina HT-12 microarray platform, and expression of target genes of interest suggested by the microarrays were further investigated using qPCR techniques. Further, bioinformatic analysis of the microarray results was carried out using typical tools such as DAVID.

Results and Conclusions: Mechanotransductive genes and pathways were demonstrably distinctive and unique between each experimental group. Only two genes were differentially expressed uniquely and in common across all scoliosis groups in response to the mechanical stress. In parallel, only 23 concretely defined genes were uniquely differentially expressed in the control group while absent in all scoliotic groups. Hierarchical clustering on genes differentially expressed in at least one of the four experimental groups produced two clusters we see as being of biological interest. These results illustrate the great diversity of mechanical response on a biological level among AIS patients.

4.2 Nomenclature

μ	dynamic fluid viscosity
ρ	fluid density
f	flow frequency (in Hertz)
τ	fluid shear stress
b or β	PPFC width
h	distance between the plates
L_c	characteristic length of the flow chamber
Pa	Pascal
Q	flow
Re	Reynolds number
α	Womersley number

4.3 Introduction

Adolescent Idiopathic Scoliosis (AIS) is the most common orthopaedic deformity found in children, affecting a significant proportion (1-3%) of them, girls from age 9-17 typically the most frequently and severely [103, 272, 273]. Although much studied, the etiopathogenesis of the disease remains unclear. Several major avenues of research have been followed, including genetics, growth hormone secretion, melatonin deficiency, and muscle structure, to name a few [1, 3, 274-278]. One of the major reasons for such a wide spread of approaches is the heterogeneous nature of the disease, both physically (curve types, patterns, locations vary immensely) and biochemically. Such variability has complicated matters immensely until now for scoliosis researchers in any aspect to present findings generally applicable and valid for the whole of the affected population. To help combat this, our laboratory has previously demonstrated the existence of three major functional subgroups of scoliosis patients into which affected individuals can be classified, based on G_i protein signalling responses [59, 60, 279].

In addition to the research axes listed above, there is another major research axis that exists, but has not been as well explored, and that is mechanotransduction. This is the process by which external mechanical loads or stimuli are converted into biochemical activity [280]. With respect to AIS development as well as its treatments (i.e. bracing), the importance of biomechanics and bodily responses to mechanical stimuli is generally well-established and agreed upon [186, 262]. Knowledge of exactly what genes and biochemical pathways are being altered because of mechanotransduction differences in scoliosis could well revolutionize the diagnosis and treatment of AIS. However, a cursory inspection of the literature reveals a distinct lack of fundamental empirical study of mechanotransduction *in vitro* in human cells and tissues, as scientists in the field seem to have relied more on animal models like chicken [281] or rat [214] to draw inference in humans. Others with a more numerical leaning have chosen to employ *in silico* biomechanical analyses of progression or treatments when considering this critical factor [188, 282-284].

While these prior studies have made valuable contributions to our understanding of mechanotransduction in scoliosis, this relative void in terms of direct examination in

humans is cause for concern as it has hampered fuller understanding of this important mechanism in the disease. A natural explanation for this paucity may be that many of the common methodologies used in such studies cannot be directly applied to humans, either practically or ethically [186]. As well, this subset of research too has been plagued by the same aforementioned disease variability preventing wider applicability of results. However, our belief is that studies of a phenomenon known as fluid flow-induced shear stress, one of the most potent modes of mechanical stimulation in the musculoskeletal system [131, 232], can help fill this gap in scoliosis research when considering the question of mechanical signal transduction.

Fluid flows over cells and their resulting shear stresses are universal to all humans across the species and are physiologically encountered due to mechanical loading in the musculoskeletal system. For instance, *in vivo*, the bone fluid existent from the region of the bone vasculature through the canaliculi to the lacunae of the surrounding mineralized tissue normally experiences a heterogeneous pressurization and depressurization due to deformation of the mineralized matrix from normal loading and unloading via locomotion of the skeletal frame, inducing shear stress from the resultant fluid flow [131]. Although this loading/unloading cycle is not usually sinusoidal, it is repetitive, leading to cycles of forward and reverse physiological fluid flow following pressure gradients [237].

There have generally been three types of fluid flow studied experimentally: steady, pulsatile, and oscillatory. Example flow rate profiles of each regime are shown in **Figure 4.1**. Looking at the figure, one will notice that oscillatory fluid flow (OFF) is characterized as a dynamic sinusoidal pattern of flow, with equal forward and reverse flow rates occurring at a given frequency over the period of oscillating flow. Oscillatory fluid flow is the most akin of the three to fluid flow in physiological settings when speaking of mechanical loading-induced flows (e.g. gait) [128, 237], and has received increasing attention from the mechanobiology community in the last 10 years since the release of a 1998 Jacobs study that was considered a landmark in the field. Typical magnitudes of fluid shear stress in the musculoskeletal system are thought to lie within a narrow range between 0.8-3.0 Pa [226, 285]. Also, fluid flows due to mechanical loading *in vivo* must necessarily be less than about 10 Hz [286]. At such magnitudes, fluid flow over cells may

seem fairly innocuous at first glance, but these levels have already been shown to be capable of causing mechanotransductive-based changes in animal studies as well as in normal musculoskeletal cells *in vitro* [287-289]. In fact, mechanical loading-induced fluid shear stresses may be an even more potent regulator of bone metabolism at typical loading magnitudes mechanical strain [234], substrate deformation [246], or streaming potentials [229].

In this study, then, we have attempted to make a first-ever examination of mechanotransduction differences in scoliosis, directly in human musculoskeletal cells by undertaking a fluid shear stress study-based approach. We believe that its universality and uniformity between individuals, regardless of other factors, makes it an excellent, equitable, and practical scientific basis of studying such fundamental differences between normal subjects and scoliotic patients, as well as finding commonalities amongst the entire spectrum of AIS sufferers. In this cause, we applied a physiologically plausible magnitude and frequency of fluid shear stress due to mechanical loading to osteoblasts sampled directly from scoliotic and control patients, and employed a broad-spectrum microarray/bioinformatics + qRT-PCR approach to extract possible early-response gene expression and pathway alterations that may be shared between or absent in all AIS patients.

4.4 Methods and Materials

4.4.1 Patient Selection

Three control subjects and nine patients severely afflicted with AIS (Cobb angle > 45^{o}) requiring corrective surgery were selected for this study. Further, the nine AIS patients were chosen in such a way that there were three patients to represent each of the three aforementioned functional classification groups of scoliotic patients that we had previously identified [290], herein referred to as Group I, Group II, and Group III. Since AIS tends to afflict young females more frequently than males (especially incidences of severe AIS, by a ratio estimated at anywhere from 5 to 10:1 in favour of females [26, 27]), all subjects and patients selected were female and aged 11-17 at the time of osteoblast sampling, in order to maintain the broadest scope and applicability of our results. Control subjects were selected from among bone trauma cases presenting at the hospital. Parents

/ legal guardians gave informed written consent to participate in this study, while at the same time, minors gave their assent. In order to preserve as much homogeneity and relevance to severe surgical cases of AIS as possible amongst the study population of scoliotics, AIS-affected patients were selected such that all possessed one of the most common curve patterns prone to curve progression, the double major curve [291, 292]. Demographics and clinical characteristics are summarized in **Table 4.1**.

4.4.2 Surgical Isolation and Cell Culture of Human Osteoblasts

In all AIS cases, osteoblasts were obtained intraoperatively during the corrective surgery prescribed to correct the scoliotic curve, from bone specimens originating from vertebrae in all cases (varying from T3 to L4 according to the surgical procedure performed befitting the double major curves); while with control subjects, bone specimens were obtained from various other anatomical sites (specifically, specimens in one control were taken from the femur, in another control from the tibia, and the last from the ankle) during surgery following a traumatic event to that site in bone which necessitated such an intervention. Bony fragments were mechanically reduced into smaller pieces via a bone cutter in sterile conditions and incubated at 37° C in 5% CO_2 in a 10 cm culture dish, in presence of α-MEM medium containing 10% fetal bovine serum (FBS; certified FBS, Invitrogen, Burlington, ON, Canada) and 1% penicillin/streptomycin (Invitrogen). After 30 days, osteoblasts emerging from the bone pieces were uniquely separated at confluence from the remaining bone fragments by trypsinization [59, 279].

These osteoblast samples were subsequently plated onto 100 mm diameter Beckman-Coulter cell culture dishes, at a density of 1 x 10^6 osteoblasts per dish, and grown to confluence in α-MEM cell culture media containing 10% fetal bovine serum (FBS) and 1% streptomycin (Life Technologies, Inc.). Following this, cells were then subcultured onto 35 mm cell culture dishes and again grown to 100% confluence before use with our parallel plate flow chamber setup. All cells were maintained in a humidified incubator at 37^{o}C and 5% CO_2. To ensure uniformity of the osteoblast population, only young osteoblasts with a passage number between P2 and P9 were employed in this study, as has been characteristic of other fluid flow experiments on osteoblasts before [289]. Once 100% confluence had been attained, the 10% FBS-containing media was replaced with

new α-MEM media containing 2% FBS and 1% streptomycin, and returned to cell culture for 24h. Finally, just before the start of flow experiments, cells were washed briefly with a 1X PBS solution.

4.4.3 RNA Isolation/Purification/Quantification

Total RNA was isolated from cells using TRIZol reagent (Sigma-Aldrich, Oakville, Ontario) as per the manufacturer's protocol, and subsequently purified in columns from RNeasy MinElute CleanUp kits (Qiagen, Toronto, Ontario). The concentration and quality of each sample was then determined by measuring absorbance at 260 nm and the 260:280 ratio using a Nanodrop 3300 Spectrophotometer (Thermo-scientific, Wilmington, DE). RNA quality was further ensured by means of finding the RNA integrity number (RIN) of each sample, using an Agilent 2100 Bioanalyzer (Agilent Technologies, Mississauga, Ontario). Taking the study as a whole, samples were not admitted for further analysis unless possessing 260:280 ratios of at least 1.8 (the lowest 260:280 ratio of any sample used was 1.86, highest was 2.33). Moreover, for microarray studies, a minimum RIN of 7.5 was necessary in order for the RNA sample to be deemed acceptable; for qPCR, the RIN minimum threshold was 7.0. A two-way analysis of variance (ANOVA) with replication was first used to compare average total RNA quantity (factors were patient class and flow condition) as a means of examining relative cellular RNA transcription activity, as in [293]. Average RNA quantity and standard deviations were reported, and p-values < 0.05 were considered statistically significant.

4.4.4 Quantitative Real-time Polymerase Chain Reaction (qRT-PCR) primer selection / Protocol

The mRNA expression of certain genes of interest (as selected either due to microarray results and/or pre-existing curiosity) was verified via qRT-PCR. Briefly, following RNA extraction, purification, and quantification, 200 μg of RNA from each sample was reverse-transcribed to cDNA using the Bioline Tetro cDNA synthesis kit (Medicorp Inc., Montréal, QC) with random hexamers as primers, according to the manufacturer's instructions. Finally, PCR amplification at an annealing temperature of 55^{o}C was performed for 50 cycles in an ABI7900HT sequence detection instrument (Applied Biosystems, Foster City, CA) by employing PerfeCTa® SYBR® Green SuperMix,

ROX™ (Quanta Biosciences, Gaithersburg, MD) together with specific primers (**Table 4.2**) designed using the NCBI web-based utility primer-BLAST [294] and obtained from Integrated DNA Technologies (Montréal, QC). Primer quality was further verified using mFold web-based software [295-297], a utility that checks for the presence of detrimental secondary structures in DNA product amplicons. Quantification of relative gene expression for COX2, OPN, and PITX1 was performed using the comparative threshold cycle (ΔΔCt) method [298]with β-actin as the internal reference, and relative gene expression was reported as $2^{-\Delta\Delta Ct}$. Fold changes of each gene were calculated in each patient and then averaged for each experimental group, such that the resulting data is presented as mean group fold change ± standard deviation. A two-way analysis of variance (ANOVA) with replication was used to compare fold changes (factors were patient class and flow condition). P-values < 0.05 were considered significant.

4.4.5 Oscillatory Fluid Flow

Cells were exposed to oscillatory fluid flow as has been previously described [128, 237]. In summary, cells grown in 35 mm cell culture dishes were directly mounted to a Glycotech parallel plate flow chamber [240], using a gasket with a rectangular fluid volume of 20 x 10 x 0.254 mm. The flow chamber was then attached to a syringe pump via rigid wall tubing, while vacuum pressure supplied by a vacuum pump held the entire apparatus together. The entire ensemble was housed in a cell culture incubator held at 37^{o}C and 5% CO_2 for the duration of flow experiments. A schematic of the experimental setup is shown in **Figure 4.2**.

Oscillating fluid flow was supplied by a 10 ml syringe mounted in an infuse/withdraw PhD Ultra Syringe Pump (Harvard Apparatus Canada, Saint-Laurent, QC). The desired sinusoidal oscillating flow profile was achieved via programming appropriate volume displacements per cycle (810 μL) into the onboard pump control software. Oscillatory fluid flow was applied for 90 minutes to osteoblast cells using a sinusoidally-varying volume flow rate of amplitude ±18 ml/min at 0.5 Hz. This corresponds to a fluid shear stress waveform of amplitude ±2 Pa at the same frequency, according to the well-established closed-form equation for rectangular parallel plate flow chambers [243]:

$$\tau = \frac{6\mu Q}{\beta h^2}, \quad (1)$$

The fluid used to expose cells to flow was α-MEM media with 10% FBS and 1% streptomycin (at 37°C, $\mu = 0.78 \times 10^{-4}$ Pa·s, $\rho = 0.99$ g/cm^3). No-flow controls for each patient sample were obtained by following the flow protocol in all aspects without activating the pump.

Equation (1) assumes steady, fully-developed laminar fluid flow between so-called "infinitely wide" plates. The verification of the truth of these assumptions in any flow experiment is important; if they are not valid, it has been demonstrated that the predictions of fluid shear stress by (1) can be extremely inaccurate [286]. Therefore, a series of checks is necessary to ensure the viability of our flow chamber design. First, for our chamber design, the aspect ratio b/h is approximately 40, thus implying that the channel width is very much larger than the channel height and so satisfying the "infinite width" requirement[286]. In addition, the Reynolds number can be calculated at 43, using the well-known equation:

$$Re = \frac{Q\rho}{\mu b} \quad (2)$$

In fluid mechanics, the transition to turbulent flow is generally accepted to take place at Reynolds numbers ranging from 2000-8000 [299], and thus our design is well within the laminar flow regime. As to the steady flow requirement, naturally a dynamic flow regime can never be "steady" in the strictest classical sense. However, as long as the Womersley number for a given flow regime is roughly less than or equal to 1, we can pronounce the flow to be *quasi-steady*, as it will largely retain the defining characteristics of classical steady laminar flow (e.g. parabolic velocity profile, a pressure gradient that is in-phase with fluid flow, etc.). The Womersley number α is defined by the following equation:

$$\alpha = L_c \sqrt{\frac{2\pi f \rho}{\mu}} \quad (3)$$

For our setup, we calculated α to be ≈ 1, satisfying another of the key assumptions. Finally, a streamwise distance known as the "entrance length" is required for the channel flow to become fully developed [300]. A conservative estimate of the entrance length for two-dimensional flow such as ours is 0.06 Re h. In our configuration the entrance length is 0.655 mm. Given that this length is just over 3% of the total channel length, the fully-developed flow assumption is clearly satisfied over the vast majority of the flow chamber window.

4.4.6 Microarray Analysis

Purified, high quality flow and no-flow condition osteoblast RNA samples from all three patients in each of the four experimental groups were submitted to microarray analysis, with the exception of the Group I flow condition, where RNA samples from two patients in this category were used (patients 1272 and 1423), making an overall total of 23 samples. Array hybridizations were carried out using the facilities of the Centre d'Innovation of Génome Québec at McGill University. 250 ng of each sample was labelled and hybridized onto Illumina HumanHT-12 v4 Expression BeadChip kits according to the Direct Hybridization Assay protocol (Illumina, San Diego, CA). All sample processing was performed in balanced batches, 12 samples per chip at a time. Microarrays were scanned under the iScan system using the native control software, and the raw data files exported in text file format.

Pre-processing of probe set data eliminated unreliable probes, defined as those not meeting a detection p-value threshold < 0.05 or the proportion per experimental group threshold > 0.5. The robust multiarray algorithm (RMA) [301, 302] was then used to analyze expression data from the remaining "trustworthy" probes on the Illumina chips. Microarray comparisons were performed on the basis of the effect of flow vs. no-flow within the same patient class (herein referred to as intra-group comparisons). Intra-group

comparisons were carried out in the Flexarray software suite v1.4[303], where fold changes and significance p-values were calculated under the Empirical Bayes (Wright and Simon) algorithm [304]. Final fold change results are reported in symmetric form (positive and negative numbers), and genes were considered differentially expressed in a comparison schema if possessing a fold change of ±3 and a p-value < 0.05. Raw data and analyzed data were MIAME-compliant[305] and were deposited in the Gene Expression Omnibus database (series accession number).

4.4.7 Clustering Analysis

Genes identified as being differentially expressed according to the fold change and p-value criteria above were further analyzed with an unweighted agglomerative hierarchical clustering algorithm using centroid linkage as the clustering method and Euclidean distance as the similarity metric in Cluster 3.0 [306]. Genes submitted to cluster analysis contained the set of differentially expressed genes identified in a minimum of one of the four intra-group comparisons. From this, we identified interesting biologically suggestive clusters of genes with similar osteoblastic expression profiles across experimental groups in response to fluid flow.

4.4.8 Functional Analysis

Lists of differentially expressed genes in each gene cluster identified as being of biological relevance as well as our lists of genes demonstrated to be commonly present or absent in all scoliotic experimental groups (with the correspondingly contrary condition in controls) were imported into the online Database for Annotation, Visualization, and Integrated Discovery (http://david.abcc.ncifcrf.gov) (DAVID)[307, 308]. The functional annotation tools available on this software were used for gene ontology (GO) classification. Also, the pathway analysis tool in DAVID was used to superimpose these lists onto the Kyoto Encyclopaedia of Genes and Genomes (KEGG) database[309] to find possible enriched pathways of interest in our gene clusters.

4.5 Results

4.5.1 RNA Analysis

The total RNA extracted from patient osteoblasts found to vary in a borderline significant manner with experimental grouping (p=0.059), but not with flow condition

(p=0.905), as seen in **Figure 4.3**. No significant interaction between these two factors was present (p=0.95). We note that the average extracted RNA quantity was greater in every one of the scoliotic groups as compared to that of controls.

4.5.2 Differential Gene Expression

Raw data sets from the Illumina Human HT-12 v4 contained information from some 47323 probe sets. After applying pre-processing criteria for probe inclusion into data analysis of detection p-value <= 0.05 and detection threshold >= 0.5, 22692 probes passed this quality control stage. Subsequently, upon applying our differential expression criteria, we identified a total of 210 probes representing genes that were differentially expressed in at least one of our four experimental groups from the intra-group analysis. The breakdown of the number of up- and down-regulated genes for each contrast is shown in **Table 4.3**. The complete list of probes and corresponding gene names for those identified as differentially expressed under intra-group analysis is given in supplemental data **Table S1**.

In comparing the degree of commonality of differential gene expression between the three scoliotic groups, we found only a very low percentage of probes representing differentially expressed genes shared. For instance, as shown in the Venn diagram of **Figure 4.4**, out of the intra-group analysis set, only six probes (representing four distinct genes, as there were two pairs of duplicate probes) were common to all three. Further, just two of those four genes (RN7SK and RIMBP3) were uniquely differentially expressed (both upregulated) across Groups I, II, and III, exclusive of the control group. Looking from the other perspective, there were also 44 genes that were differentially expressed in only the control group, of which 23 were such with well-defined gene names and functions (i.e. not predictions of a gene's existence) as listed in **Table 4.4**, up- or down-regulation indicated by colour and classed by function where possible, as returned by our analysis in DAVID.

4.5.3 Hierarchical Clustering/ Functional Analyses

We identified two interesting sub-clusters from the gene set in the intra-group analysis whose dendrograms are illustrated in **Figure 4.5**. Both selected sub-clusters possessed correlations > 0.80. There were a total of 18 distinct genes in the first intra-

group analysis sub-cluster with a correlation factor of 0.820, and 14 in the second (correlation = 0.857). Selected gene groups of interest assembled from among the genes in these respective clusters and classed together on the basis of our functional/pathway analysis of these clusters are shown in **Tables 4.5a** and **4.5b**, including mechanical stimulus response, calcium signalling, G-protein coupled receptor signalling, multicellular organismal development, and chemokine signalling. Interestingly, all genes involved in chemokine signalling were also found to be part of NOD-like receptor signalling pathway from KEGG pathway analysis.

4.5.4 Expression of PITX1, COX2, OPN

qRT-PCR was performed to measure the expression of three genes: pituitary homeobox 1 (PITX1), cyclooxygenase-2 (COX2), and osteopontin (OPN). PITX1 expression was relatively unchanged by our applied fluid shear stress across all experimental groups, with no fold changes greater than 3-fold in any according to qRT-PCR analysis (**Figure 4.6**, **Panel A**). In contrast, average qRT-PCR COX2 expression was up-regulated by more than 3-fold in all experimental groups, particularly in groups II and III where a 33.5 and 26.9-fold change, respectively, was observed, though with very large internal group standard deviations (**Figure 4.6**, **Panel B**). Average osteopontin expression measured by qRT-PCR showed little change in controls and group I scoliotics, but did exhibit greater than 3-fold upregulation in Groups II and III (3.34 and 3.43, respectively) (**Figure 4.6**, **Panel C**).

For comparison's sake, corresponding fold changes of flow vs. no flow conditions in each experimental group for these genes according to our microarray procedures are presented in **Table 4.6**. We note that for all three genes under consideration, there was some degree of divergence between the expression fold change values shown by each technique employed.

4.6 Discussion

In this study, we saw, for the first time, the use of fluid-induced shear stress as a means of comparing mechanical loading-induced mechanotransduction in scoliosis patients versus unaffected individuals *in vitro*. This involved the use of a parallel plate flow chamber setup to supply such shear stress at physiologically typical levels to cultured primary osteoblast cells obtained from human subjects representing unaffected

individuals as well as the range of functional classifications of scoliosis patients as previously defined in earlier works of our own [290, 310]. Principally, we found that the mechanotransductive gene expression response of every group, scoliotic or otherwise, was to a large extent distinct from every other, as evinced by the very small percentage of genes we determined to be commonly differentially expressed between them. As well, through hierarchical clustering performed on the set of genes identified as differentially expressed in at least one experimental subgroup, we were able to derive at least two biologically interesting clusters. A number of gene groups found inside these clusters via our functional/KEGG pathway analysis already known as important players in mechanotransductive response were illuminated, as were several novel gene groups whose relation to mechanotransduction is not yet clear. Finally, through qRT-PCR, the expression levels of certain genes, namely PITX1, COX2, and OPN, were each found to be altered in some or all of the experimental subgroups in this study, although results returned from our microarray and qRT-PCR analyses did not always agree reciprocally in all cases.

4.6.1 Commonalities among Scoliotic Groups

We found only two concretely-defined genes (i.e. not predicted) commonly differentially expressed in all scoliosis groups but not in controls from our intra-group analysis, namely RIMBP3 and RN7SK. Both are relatively new discoveries in genomics itself, whose full biological function is as yet only partially understood. The former, RIMBP3, or RIMS binding protein 3, is a molecule that has recently been enumerated as being potentially important to sperm motility [311]; however, no former association with mechanotransduction or the musculoskeletal system has ever been reported, so its presence in this study may represent a novel finding. The latter, RN7SK, is a small nuclear RNA gene that aids in the transcription of RNA in conjunction with P-TEFb [312]. As we determined a universal upregulation across scoliotic groups in our screening, one might expect a commensurate increase in RNA production compared to controls. However, our ANOVA analysis of RNA quantification showed that while RNA quantity was greater among scoliotic groups in a borderline significant manner, this disparity was due more to the differences in experimental groups themselves without reference to any flow effects.

Thus, we suggest that RN7SK may well be performing another, more novel biological role relevant to mechanotransduction and scoliosis as a result of fluid shear stress, since our evidence seems to suggest that the upregulation due to applied fluid shear stress does not serve to increase RNA transcription in scoliotic cells.

Among the 23 concretely-defined genes exclusively differentially expressed in controls, a significant portion of them were as yet not well enough understood to assign biological function in DAVID. Nevertheless, we noted a large fraction of the up-regulated whose function relates to interaction with or transport of cellular ions and solutes of varied types, particularly zinc, as seen in **Table 4.4**. Variations in zinc levels themselves could be very crucial, as such changes have been observed concurrently with scoliosis as well as other bone malformations in several animal models [37, 313] and in humans [40]; although, to be fair, some have intimated that this might simply be a secondary consequence of the presence of scoliosis rather than a primary causative agent [314]. As a collective whole, however, solute and ion transporters may well be involved in normal mechanotransductive-linked bone remodelling on a general level by helping to balance ion levels and bringing necessary ingredients to osteoblasts to facilitate matrix protein synthesis later [293], the alteration/interruption of which may thus play a role in AIS pathogenesis. At the individual gene level, there were a couple of such suggestive occurrences of alterations observed with possible ramifications for AIS. First, CALCA is a gene that aids in regulation of cellular calcium ion homeostasis. Having already witnessed changes in elements of zinc ion regulation, it is perhaps not unreasonable to expect observable changes in calcium ion regulatory mechanisms as well, especially since zinc is in fact a necessary component for calcium metabolism to proceed properly [315]. Now, calcium interactions have implications in AIS due to its close function with the inflammatory cytokine osteopontin, which we have previously demonstrated in AIS as a disease induction/progression indicator [97]. Ca^{2+} flux and signalling is a known cellular response to mechanical force [316], and is a required precursor event to subsequent OPN upregulation [128]. Then again, there was also a strong presence of genes coding for selenoproteins, represented by SELS and DIO3. Overall, selenoproteins are required for many fundamental cellular processes, and either in turn require selenium for initial

synthesis or bind it as a cofactor [317]. As far as selenium concerns AIS, not only is it of interest since plasma selenium concentrations were recently confirmed to be significantly lower in those affected with the disease [38, 39], but also environmental exposure to high levels of selenium has just been identified as a possible scoliosis risk factor [318]. Speaking specifically of the two identified selenoproteins in this list, well-documented pathologic conditions arising from primary deficiency of either in humans have so far not been reported. However, SELS does seem to play a role in inflammatory response through control of cytokine production in macrophages [319, 320]. Knockout of DIO3 in mice presented severe developmental defects [321], implying perhaps at least some assignment in growth processes. Quite fascinatingly as well, the suppression of the selenoprotein SELS *in vitro* was shown to increase the release of the inflammatory cytokine IL-6 in healthy subjects in a recent study [320], indicating a role in control of inflammatory response. Our results agree in this regard, as it happens that IL-6 is also part of our list of up-regulated differentially expressed genes unique to controls. Observing the absence of this axis between SELS and IL-6 in any of the scoliosis groups, we propose that this may hint at an important element of normal mechanotransduction response gone awry in AIS.

4.6.2 Hierarchical Clustering

We performed gene cluster analysis in order to identify potentially novel relationships between identified differentially expressed genes. Interestingly, several gene groups in our two clusters of concern, in particular FOS/JUNB, calcium signalling, and chemokine signalling, are consistent with known early-response mechanotransduction gene groups determined in a previous study employing traditional cyclic compressive loading on rat hindlimbs [293], indicating support for the concept that fluid flow may actually be a means for such conventional loading of effecting at least some of the biochemical changes that the literature typically attributes to conventional loading alone. It is known that gene expression of members of the FOS and JUN families increase in response to mechanical loading, including fluid shear stress [245, 322, 323]. Generally speaking, that was the case here as well with FOS and JUNB, though not to our defined

threshold of differential expression in all experimental groups. Others have observed increase expression of these family members at the protein level [324]; it may be worth confirming this effect in our setup in future work.

Chemokines figured prominently in both clusters. These molecules are known to be important in development as well as inflammatory responses such as immune response and wound repair [325]. Many chemokines in the immune system act as regulators of osteoblasts [215], and osteoblasts produce both chemokines and their corresponding receptors [121]. The genes in our clusters classifiable as chemokines included an interleukin family member (IL8), a couple of C-X-C motif ligands (CXCL1 and CXCL2), and a C-X3-C motif ligand (CX3CL1). Although both IL8 and CXCL2 were strongly differentially up-regulated genes in all experimental groups, they were more so in each scoliotic group as opposed to the control group, with the exception of IL8 in Group II patients, where the fold change threshold was not met (marginal upregulation). Overall, given that our results show fluid shear stress activating all manner of inflammatory response molecules and pathways, it is provocative that levels of both IL-8 and CXCL2 are known to be elevated in inflammatory diseases that also exhibit progressive bone loss, such as rheumatoid arthritis, osteoarthritis, osteomyelitis, spondyloarthropathy, and periodontal disease [326-329]. The other two, CX3CL1 and CXCL1, were returned by our DAVID analysis as functional in G-protein coupled receptor binding. Our group has previously expounded upon G-protein differential inhibition in scoliosis, in concert with melatonin signalling [59, 279, 290, 310]. Some of the most essential G-protein modulators are protein kinases, in particular members of the protein kinase C family, whose own catalytic activities are altered by tyrosine phosphorylation. Incidentally, protein kinase C binding and tyrosine phosphorylation functions are precisely some of those ascribed respectively to the genes PDLIM5 [330, 331] and DUSP2 [332, 333], also found this cluster.

Finally, we uncovered two groups of genes, representing response to stimulus and multicellular organismal development according to DAVID, which may constitute novel findings in mechanotransduction and AIS. The former, response to stimulus, contained the

genes SERPINB2 and C1S. Members of the SERPIN family have been found mechanoinducible in a previous study, thought to be involved in extracellular matrix formation and remodelling [293]. C1S, on the other hand, does not have an existing reputation as mechanoresponsive. However, like CALCA, C1S does possess a function in calcium ion binding, so perhaps its identification should not be so surprising. Moreover, at least in rats, a link was established between expression of a C1S homolog and cAMP signalling [334], which plays a role in G-protein signalling. The latter group comprising genes involved in development contained SHISA2, KLF10, and APOLD1. APOLD1 and SHISA2 are relatively uncharacterized genes in humans; however, a homolog in mice was found to be antagonistic to WNT signalling [335], a pathway that plays an important role in bone cell differentiation, proliferation, apoptosis, and bone formation in response to mechanical loading [336]. Also, APOLD1 expression was increased in cardiac tissue of rats exposed to acute physical activity [337], a change that may be attributable to increased fluid shear stress on the myocardium resulting from higher blood flow rates. KLF10, by contrast, has been extensively annotated and assigned a number of capabilities. Its overexpression in human osteoblasts can mimic the activity of TGF-β, regulating bone growth and metabolism [338], while KLF10-knockout mice display correspondingly decreased cellular levels of the so-called osteoblast master gene RUNX2, as well as severe osteopenic phenotypes with defects in both cortical and trabecular bone that fascinatingly are specific to females [339]. This gender-specificity is highly reminiscent of AIS. Further, a recent study in 2009 examined gene expression profiles in a dozen osteoporotic patients and identified KLF10 as a potential target in the disease, being one of 150 genes whose expression in skeletal tissues differed markedly between osteoporotic and nonosteoporotic patients [340]. Clearly, this is a gene with a pedigree in bone disorder, one that may warrant further attention in AIS research.

4.6.3 qRT-PCR Validation

Our qRT-PCR experiments served to confirm and further examine the expression of COX2, PITX1, and OPN. The first, COX2, was already of some interest, as it is a known bone cell activator [175, 202] and mechanosensitive molecule [341], particularly to fluid shear

stress [270]. Our microarray and qRT-PCR analysis agreed in that both showed greater than 3-fold upregulation in all experimental groups. However, in groups II and III, our qRT-PCR fold change values were much, much higher than those returned by microarray analysis. This hyperactivity of COX2 with respect to controls and group I might be an indication of a heightened mechanosensitivity in these patients, although one must be cautious in drawing conclusions due to the conflict of results between the two techniques. However, such an interpretation would be consistent with the pattern of OPN expression as displayed through qRT-PCR, which paralleled COX2 in showing differential expression in groups II and III, whilst controls and group I expression was relatively unchanged. This gene was of great interest to us in a number of ways. First, we ourselves previously showed an association between elevated levels of OPN in the body and increased risk of scoliosis [97]. More than that, though, OPN is known to be a mechanoresponsive molecule [128, 342-344], documented numerous times *in vitro* as a key responder to fluid-induced shear stress. Interestingly, however, it generally was a steady-state responder in those studies, showing mRNA up-regulation in response to fluid shear stress only after at least six or more hours following cessation of flow [137, 345]. Recalling that in the present case we elected to study fluid flow effects immediately following flow stimulation, the observed upregulation is all the more surprising; it may hint at a heretofore unknown alteration in signalling of this molecule in these AIS patients. Indeed, together with the previous discussion in the introduction of the capability of mechanical loading to induce fluid flow in the musculoskeletal system, these data may provide a possible explanation as to why OPN elevation and increased scoliotic risk might occur *in vivo*, at least in Group II and III patients. Such a notion fits nicely with our own clinical observations of OPN in patients, as Group II and III patients generally possess the highest OPN levels whereas controls' and Group I patients' readings remain somewhat lower. Finally, PITX1 expression was found to be downregulated in Group III patients over 3-fold according to microarrays, though it did not meet the p-value significance criterion ($p \leq 0.05$). Now, solely on this basis, this normally would not have induced us to look any closer at this particular gene. However, our attention was already drawn to PITX1 since it was discovered in our lab to play a key role in osteoarthritis, a degenerative bone disease, where lack of PITX1

expression in cartilaginous tissue leads to degradation of discs and joints characteristic of the malady [346]. Upon closer inspection of the microarray data, we then also noted an almost 2.5-fold downregulation in Group I patients as well, though again not meeting the significance criterion. Were it a single case of such measurements, one could comfortably attribute it to being a random anomaly; the fact that this downregulation occurred in two instances (groups), though, makes it difficult to disregard as a mere aberration. Concomitantly, the consequences of such an alteration in expression could be enormous in AIS. In the first place, there are instances of osteoarthritis and scoliosis occurring together in the same patient. Many times, this happens in the guise of disc degeneration in the spine via a process that causes degradation reminiscent of the deterioration experienced by other joints and cartilaginous tissue due to osteoarthritis elsewhere in the body [277]. Moreover, disc deformity, either through mechanical loading or otherwise, has been advanced in recent years as a possible mechanical contributing factor in scoliosis induction and/or progression [261, 347-349]. Our PITX1 microarray results, if true, could thus complement this theory by suggesting an elegant mechanism of said disc degradation in certain AIS patients. For if one considers the anatomy of the intervertebral disc and spine, one finds the entire construction to be highly hydrated, full of liquid. Mechanical loading of this structure is capable, then, of subsequently inducing fluid flow and associated shear stresses, causing downregulation of PITX1, which could in turn lead to the same weakening defects in the constitution here as we observed previously in joints and cartilage afflicted with osteoarthritis, ultimately encouraging the scoliosis phenotype. It is a fascinating concept, though nevertheless, of course, one that should be supported by further fluid shear stress studies of similar design, ideally performed in chondrocytes, the primary cell type in spinal discs [277, 350].

We note that microarray screening and qRT-PCR techniques did not always return concordant fold change values for each experimental group where both were employed to measure expression of the same gene. Indeed, the previous discussion of possible implications of altered expression of PITX1, COX2, and OPN is predicated in many places on fold change values that differed between techniques. The discrepancies in the expression results in these circumstances could be explained by cross-hybridization of the

probes on the arrays with other targets, or possibly the difference in probe locations. The underlying reason for the discord caused by different detection regions may be associated with the degree of accuracy in the sequence information being varied at different regions, due to the continuous discoveries of previously unknown SNPs and spliced variants. Hence, even if the primers or probes from different technologies recognize the same region, there is a possibility that they may detect different spliced variants or transcripts with different SNPs. For all these reasons, we freely acknowledge the difficulties and intend that those implications discussed rest as suggestive possibilities rather than hard conclusions, as the disagreement between techniques renders it impossible to either accept them wholeheartedly or dismiss them out of hand.

4.6.4 Limitations/Future Directions

Some other, more general limitations of this study must also be acknowledged. First, the fluid flow stimulation considered in this design was two-dimensional in nature. Bone and other musculoskeletal structures, though, exist in 3D, and elsewhere there has been some evidence of differences in behaviour, either genetic or otherwise, depending on existence in a 2D or 3D environment [265, 285, 351, 352]. However, this should not completely invalidate our results any more than it would other 2D studies in the literature; they should still serve as a useful guide. Second, for the sake of simplicity we examined just one frequency of fluid flow in this work, 0.5 Hz, since we were considering mechanical-loading induced fluid flow, which must necessarily be less than 10 Hz in frequency (generally referred to as low-frequency flows) [286], as mentioned in the introduction section. Real physiological *in vivo* fluid flows in the musculoskeletal system, on the other hand, can be much more complex, comprising several spectral frequency components in concert including some much higher than 10 Hz. For example, small strains (< 10 με) in bone show strain information extending to 40 Hz [353]. Theoretical extrapolation predicts that strain induced flow in bone elicits shear stresses up to 3 Pa for 100-200 με at 20-30 Hz [233]. Indeed, frequencies as high as 75 Hz were demonstrated in human gait [201, 354]. High frequency modes have been shown to be capable of being stimulatory to cells [202, 203, 205, 355], so future fluid shear stress studies should ideally be extended or inclusive of these elevated ranges of frequency in their design in order to

explore the fuller picture of its effects in the musculoskeletal system. Third, this work was restricted to early-response gene expression/pathway alterations. In that cause, we observed these changes through extraction of mRNAs immediately after cessation of flow. However, as previously alluded to already somewhat, particularly in discussion of OPN expression, earlier works concerning fluid shear stress tended to focus on so-called steady-state response, implying observation of effects only after return to culture for several hours [137, 345]. As well, the length of time of fluid shear stress stimulation was also variable [216, 344, 356]. Recalling that AIS is a long-term disease that is generally quite slow in developing, as valuable as early-response studies are, examination of steady-state responses with longer periods of fluid shear stress stimulation (or some intermediate combinations of these parameters) must be considered worthwhile to AIS mechanotransductive understanding. Finally, as regards the use of osteoblasts in this study, it was generally favoured for its relative ease of acquisition and usage. It must be noted, nevertheless, that osteoblasts are but one cell type among many in the musculoskeletal system that could play a role in mechanotransduction and/or AIS (e.g. chondrocytes, myoblasts, osteocytes, etc.). We have already remarked how future study in chondrocytes of this genre may prove fruitful as it pertains to PITX1. Furthermore, although more or less every musculoskeletal cell type is believed to possess mechanotransductive capabilities to some degree, osteocytes are generally the consensus choice in the literature to be the most likely cell type to act as mechanosensors and drive overall mechanotransductive response [218]. Here, owing to the practical difficulties of handling and using osteocytes in this context, we elected to use osteoblasts instead. It must be acknowledged that certain differences between the two types' responses to fluid flow have been advanced [357]. We would like to point out, however, that as osteoblasts are actually parent cells of osteocytes, one might still reasonably expect to learn a fair amount from the former about possible responses in the latter, due to this shared lineage.

4.7 Conclusions

Fluid shear stress is a potentially rich region of mechanotransduction research today, with much unexplored terrain yet to be discovered in relation to scoliosis. It is a field whose importance has already been recognized elsewhere but seemingly neglected

hitherto with respect to AIS. Inasmuch, its advent has permitted us here to make the first ever *in vitro* study of mechanotransduction in human tissues in scoliosis to date, to our knowledge. We believe we have provided seminal insight into basic mechanotransductive transmission differences in scoliotic patients as opposed to unaffected individuals, as well as sufficiently illustrated the significance of fluid shear stress in AIS musculoskeletal dynamics. Looking ahead, we hope to have turned some measure of attention of the scoliosis scientific community towards the possibilities of this phenomenon. Its use may allow researchers to acquire information about mechanotransduction in AIS directly in human cells/tissues that would be otherwise unobtainable, due to the practical and ethical difficulties mentioned before.

4.8 Acknowledgements

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Dr. Moreau reports having received from Fourth Dimension Spine LLC research grants and served on the scientific advisory board of Fourth Dimension Spine LLC. No other potential conflict of interest relevant to this article was reported.

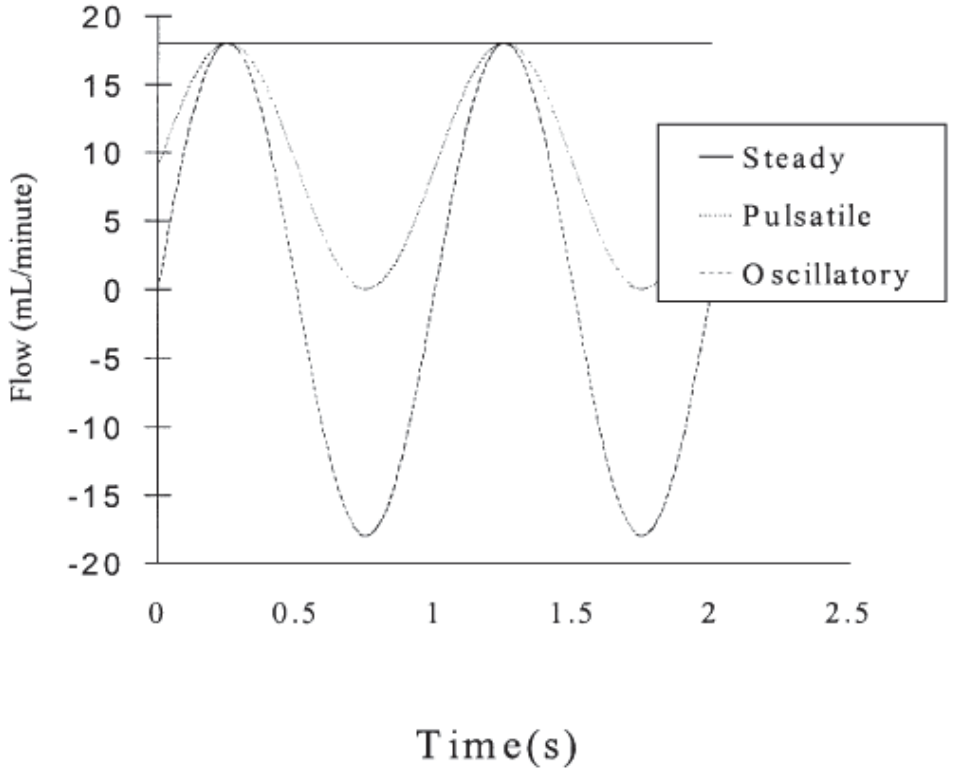


Figure 4.1. Representative flow profiles of steady, pulsatile, and oscillatory regimes. Note the alternating, sinusoidal nature of oscillatory flow, the regime employed in this study.

Table 4.1. Demographic and clinical data of patients selected, with age and Cobb angle.

Patient Random #	Experimental Group	Age	Cobb
19	Control	15,5	n/a
11	Control	15,71	n/a
12	Control	14,23	n/a
1423	Group I	14,57	73-45
S-1335	Group I	17,6	47-50
1272	Group I	11,65	63-39
1042	Group II	14,24	70-48
1122	Group II	13,41	60-48
1029	Group II	14,4	56-53
1074	Group III	13,28	59-57
S-1420	Group III	13,42	60-48
S-1373	Group III	14,61	41-48

All patients were females aged 11-17, with double major curves of Cobb angle greater than 45 degrees.

Table 4.2. qRT-PCR primers used in this study, 5'-3' end.

Gene	Forward Primer	Reverse Primer	Amplicon Length (bp)	Position
OPN	ATGATGGCCGAGGTGATAGTG	CAACTCCTCGCTTTCCATGTG	128	632-759
PITX1	GAGGTCCATCTCAGAACAC	CAGTCCAACATACACAGGG	236	2076-2311
COX2	ATGTTCCACCCGCAGTACAG	AGAAGGGCAGGATACAGCTC	208	1411-1618
β-Actin	GGAAATCGTGCGTGACAT	TCATGATGGAGTTGAAGGTAGTT	233	702-934

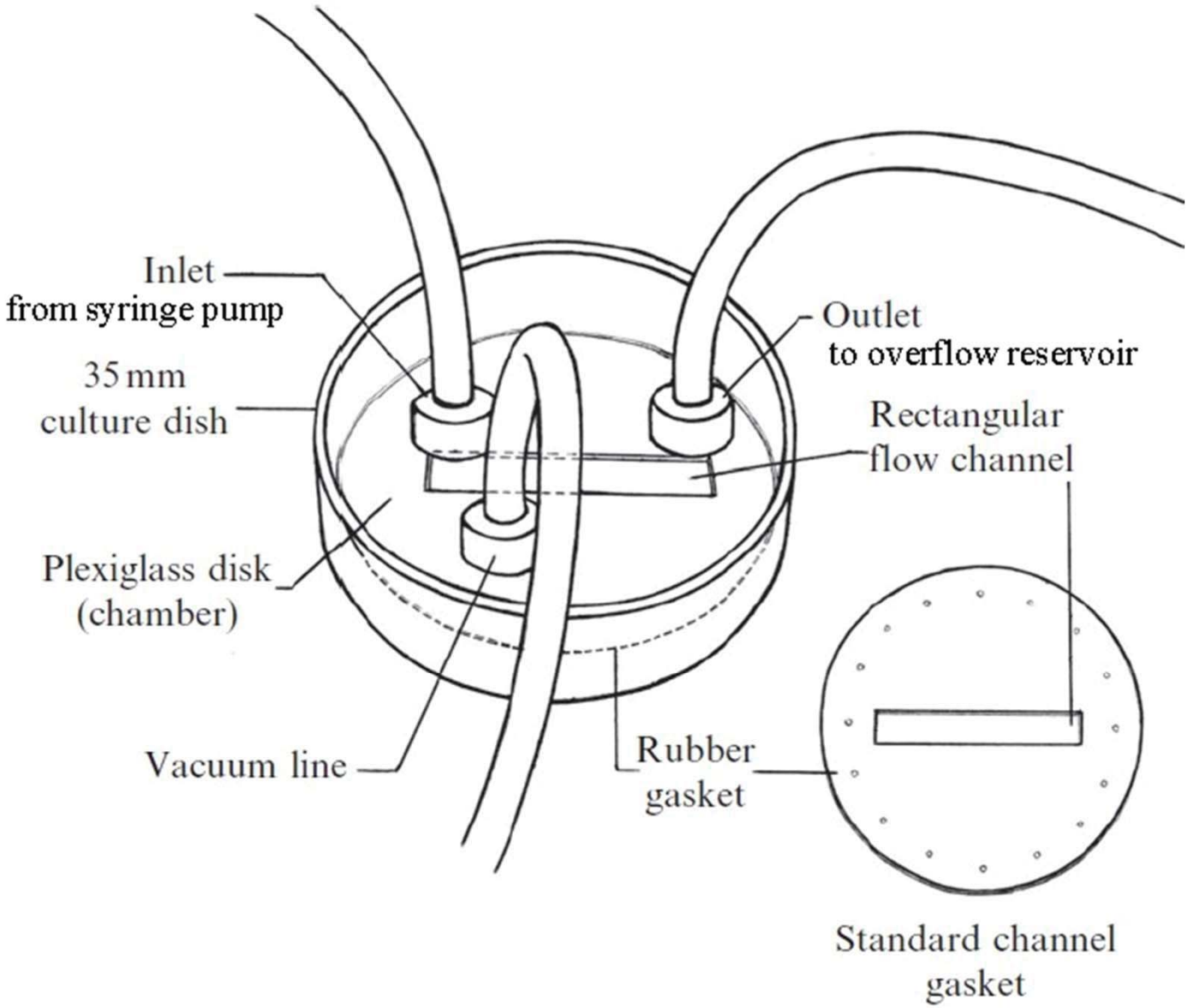


Figure 4.2. Parallel plate flow chamber design schematic and setup.

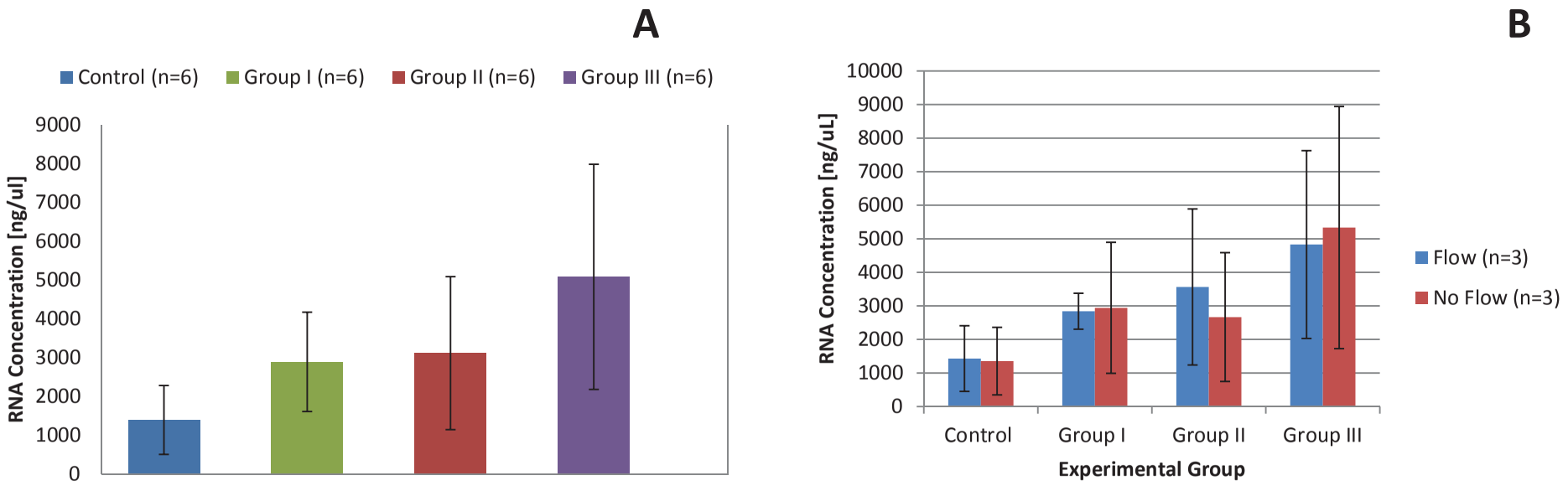


Figure 4.3. Analysis of average total RNA extracted in each experimental group. According to two-way ANOVA analysis (experimental group and flow condition as factors), the amount of RNA extracted varied with experimental group (**A**) in a borderline significant manner (p=0.059), but was not dependent on application of flow (shown in **B**, p=0.905). No significant interaction was observed between the factors (p=0.95). We note that average extracted RNA quantity was greater in every scoliotic group compared to controls.

Table 4.3. Number of differentially upregulated and downregulated genes in each group due to flow, after pre-processing of microarray data.

	CTRLFvsNF	GroupIFvsNF	GroupIIFvsNF	GroupIIIFvsNF
Upregulated	40	98	17	32
Downregulated	17	20	14	7

Criteria for differential expression were: |Fold change| ≥ 3, significance p-value < 0.05.

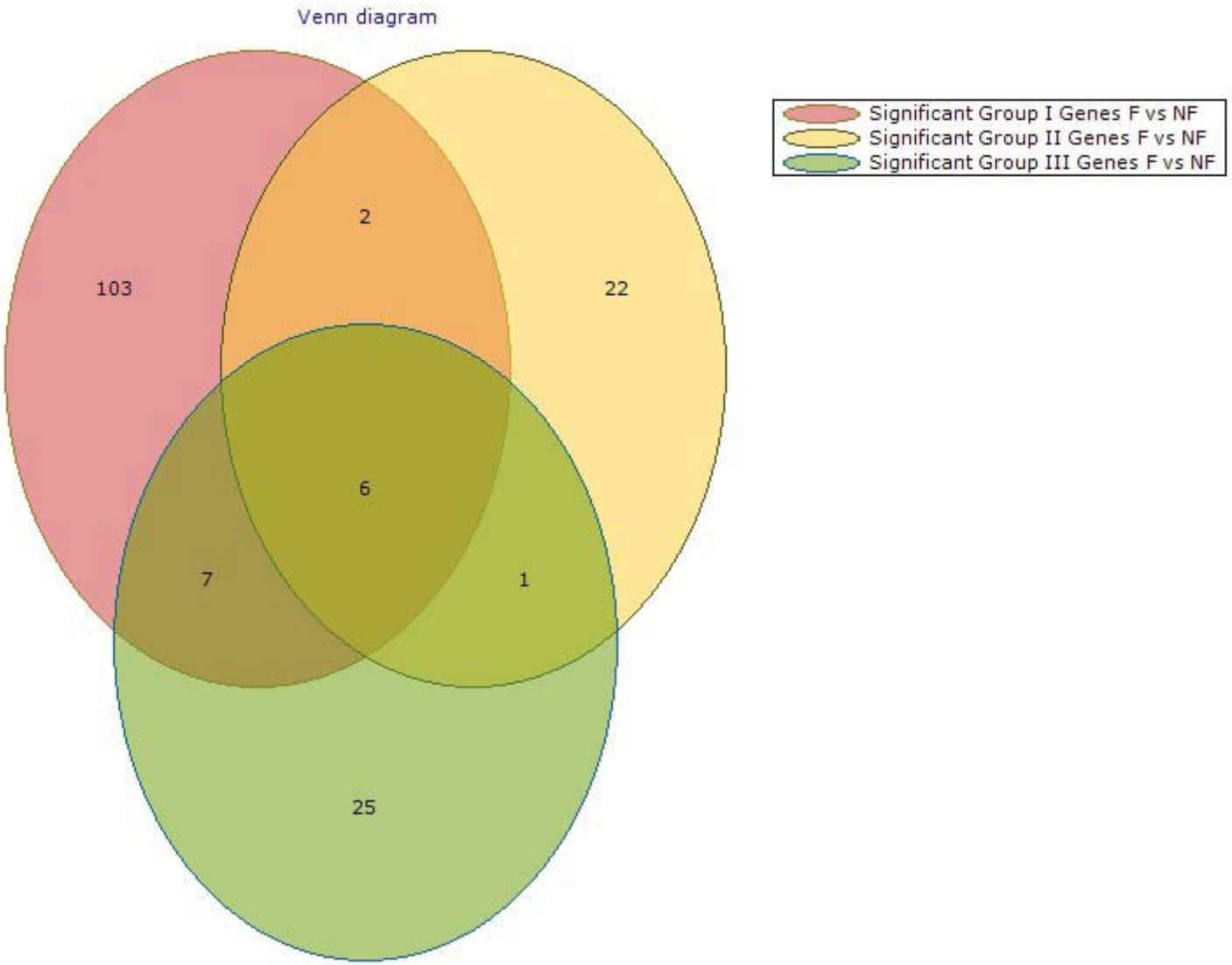


Figure 4.4. Venn Diagram of overlap of probes representing differentially expressed genes in the three scoliotic groups. Only six probes, corresponding to four distinct differentially expressed genes were found in common across all three.

Table 4.4. Well-defined genes differentially expressed (according to our criteria) in controls due to flow, but not so in any scoliotic group.

zinc ion binding
TRPS1
AIM2
ZNF568
BIRC3

ion channel activity
GABRB1
MCOLN3
SLC40A1
ZP3

Selenium binding
DIO3
SELS

organismal development
IL6
CALCA
CHST2
SF3B1
PBRM1

miscellaneous
C16ORF38
TTC39B
HEBP2
SNORA76
U2AF1
GMPR
RNY5
FIGNL2

Genes in green denote significant up-regulation; red indicates significant down-regulation of expression. In total, we observed 23 such genes as listed above, with major biological functions returned by DAVID analysis such as ion transport/activity, selenium binding, and organismal development.

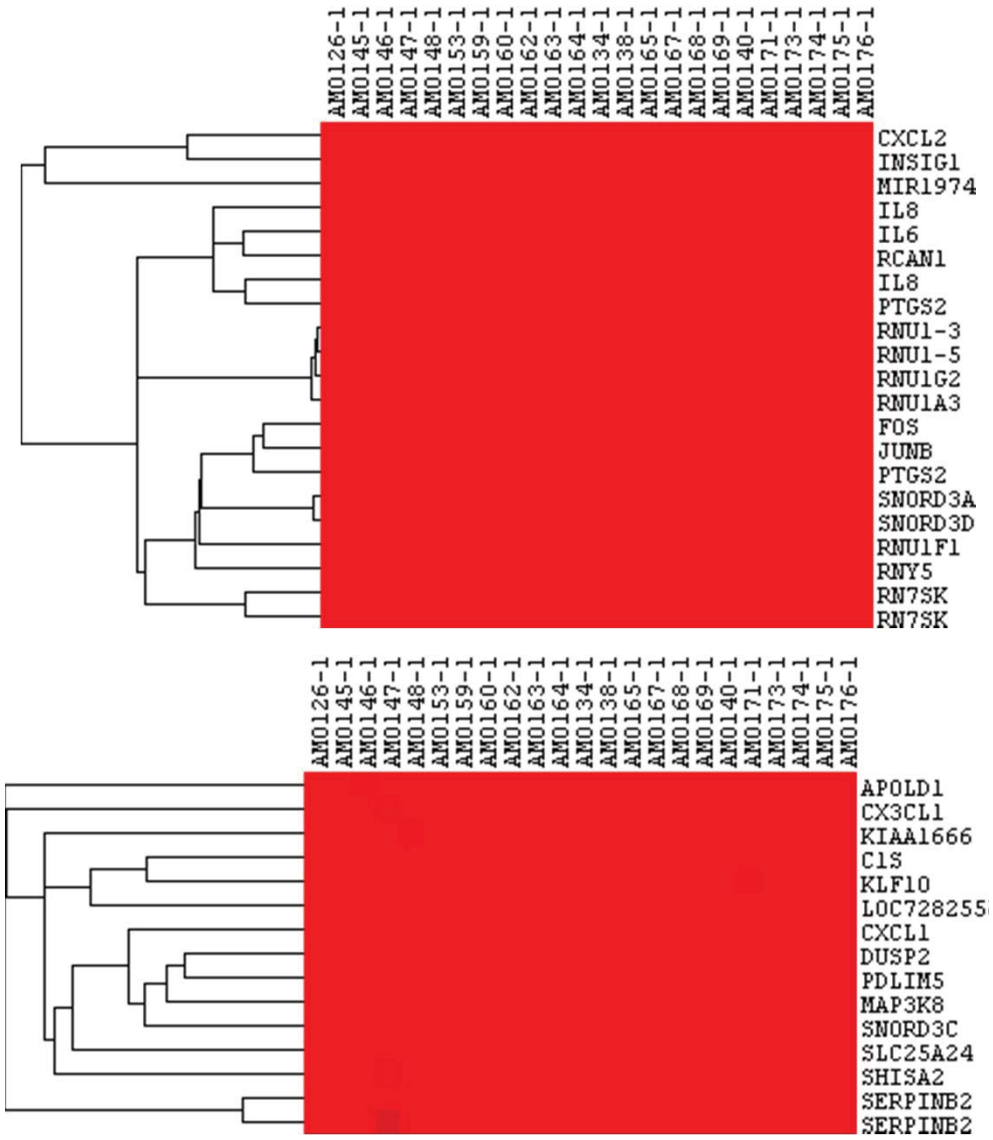


Figure 4.5. Dendrograms of hierarchical clusters identified as being of biological interest. Both clusters possessed correlations > 0.80.

Table 4.5a. Selected gene groups of interest from clusters in **Figure 4.5**, **Panel A**.

Response to mechanical stimulus
RCAN1
FOS
JUNB

Regulation of inflammatory response
IL6
PTGS2

Chemokine-chemokine receptor signaling /NOD-like receptor signaling pathway
CXCL2
IL8

Table 4.5b. Selected gene groups of interest from clusters in **Figure 4.5**, **Panel B**.

G-protein coupled receptor binding
CX3CL1
CXCL1

Protein kinase binding
PDLIM5
DUSP2

Response to external stimulus
SERPINB2
C1S

Multicellular organismal development
SHISA2
KLF10
APOLD1

Genes above are classed according to functional/pathway analysis in DAVID. It is interesting to note that genes classified above in the left column are known players in mechanotransduction, via the function suggested by their respective classification headings in italics.

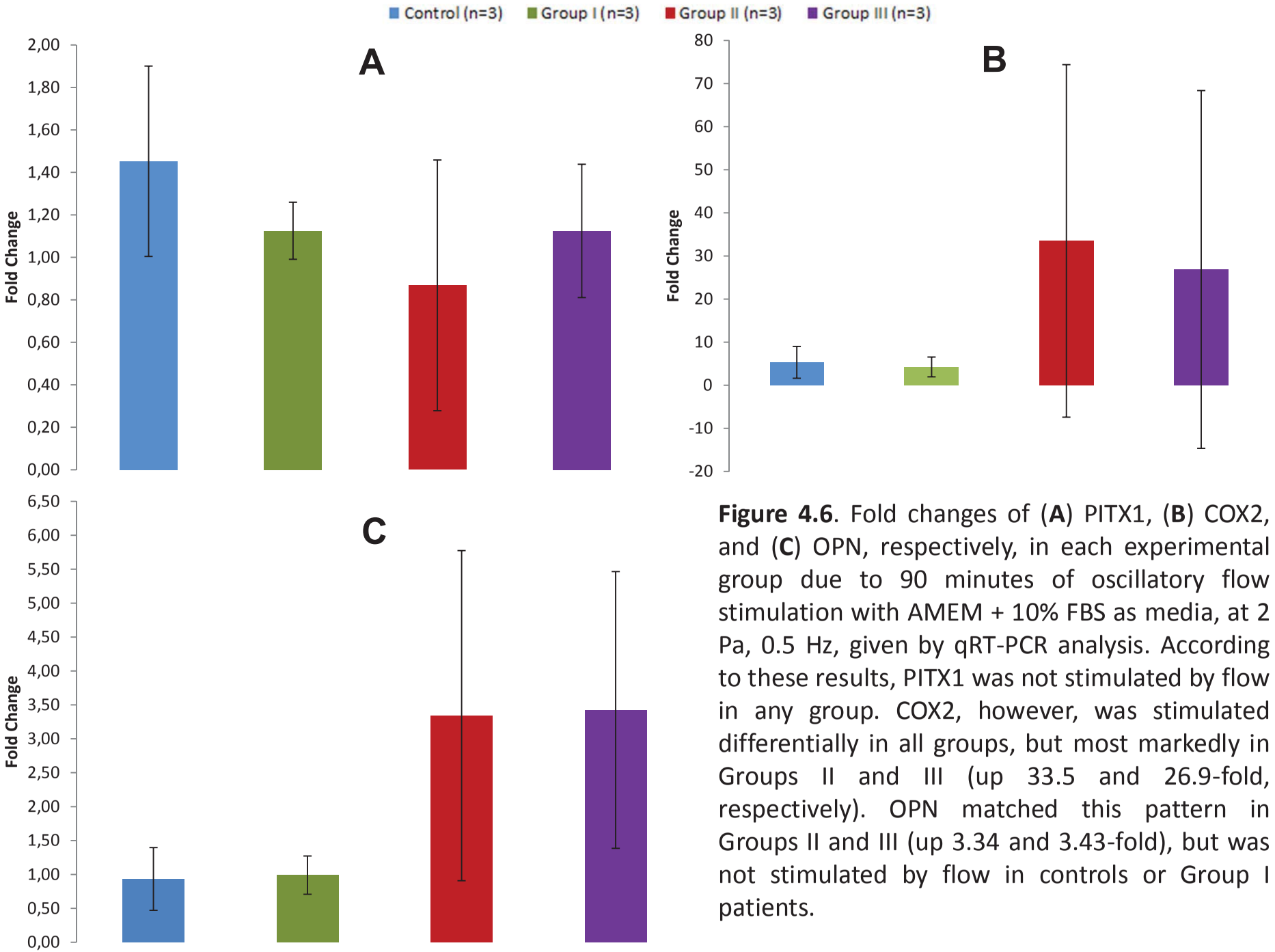


Figure 4.6. Fold changes of (**A**) PITX1, (**B**) COX2, and (**C**) OPN, respectively, in each experimental group due to 90 minutes of oscillatory flow stimulation with AMEM + 10% FBS as media, at 2 Pa, 0.5 Hz, given by qRT-PCR analysis. According to these results, PITX1 was not stimulated by flow in any group. COX2, however, was stimulated differentially in all groups, but most markedly in Groups II and III (up 33.5 and 26.9-fold, respectively). OPN matched this pattern in Groups II and III (up 3.34 and 3.43-fold), but was not stimulated by flow in controls or Group I patients.

Table 4.6. Fold changes returned by Illumina HT-12 v4 microarrays for genes also tested by qRT-PCR.

Gene	Controls F vs NF Fold Change	Group I F vs NF Fold Change	Group II F vs NF Fold Change	Group III F vs NF p-value
PITX1	1,12	-2,11	1,14	-3,22
COX2	5,25	4,37	3,69	5,13
OPN	1,09	1,76	-1,14	-2,34

We observe deviations in values from qRT-PCR analysis in many instances; PITX1 shows notable downregulation in groups I and III; OPN is mostly unchanged, though showing some downregulation in group III. COX2 agrees somewhat with qRT-PCR, though expression in Groups II and III do not display the sharp upregulation shown by qRT-PCR.

Table S1. Supplemental data table giving intra-group fold change values of each gene and probeset for flow vs. no-flow conditions. Fold change values higher than 3 are highlighted in green; those lower than 0.333 (i.e. -3 symmetric fold change) are highlighted in red.

Probe ID	Target Gene Name	Fold change CTRL F vs NF	Fold change Group I F vs NF	Fold change Group II F vs NF	Fold change Group III F vs NF
610093	ACADVL	1,349557	**3,354501**	0,4158641	0,806318
1260270	AIM2	**0,2796783**	1,343653	0,7681165	1,137847
2680220	AK5	2,027829	**4,262362**	1,032415	0,775955
4230156	ALB	0,5014252	**4,540191**	2,656389	2,828516
5560670	ALG1	1,610594	0,7100124	**0,3192399**	0,7222194
1770364	APOLD1	0,6330021	**3,655609**	2,737071	2,013939
4780010	ARL4D	1,365605	**0,3128503**	1,101433	1,683205
7000609	ARRDC2	0,7508482	0,8533066	0,8453995	**0,2682317**
5080021	BIRC3	**5,580972**	1,85433	2,08103	2,087403
6270452	C15ORF23	0,8067243	**3,227958**	0,7973657	1,09106
5890382	C16ORF38	**3,04969**	1,503716	0,5713902	0,9591998
6400114	C17ORF59	0,8405992	**0,3203962**	1,539505	1,027176
4900471	C1ORF177	0,8201765	**3,041183**	1,351922	1,278833
1300327	C1S	1,579256	1,796813	**0,2776214**	0,6673046
6980167	C21ORF121	0,9348825	1,429254	1,238626	**3,096309**
2710538	C6ORF134	1,383775	**0,2763227**	0,9975986	0,6889709
5130441	CA5BP	1,081057	**3,616405**	1,151704	0,9498988
6110242	CALCA	**3,047526**	1,14863	0,8727272	1,081781
4230292	CD109	1,170864	**0,3292365**	1,345027	0,7420432
540692	CHST2	**3,048291**	0,9848233	0,8959584	1,373367
1230259	CLEC6A	0,6795267	**3,107949**	0,7343844	1,263506
2030386	COPG2	1,545043	1,082083	**3,101426**	1,275435
3940458	CRYL1	0,6577473	0,5500627	1,167187	**0,302598**
650445	CTSE	0,8661063	**3,410867**	1,11413	0,824998
4290554	CTSL3	1,194898	**3,238**	1,055769	1,185253
630521	CX3CL1	1,184128	**0,2884701**	0,5441704	1,161454
5720039	CXCL1	2,982596	**4,520452**	0,8945335	2,109107
4670390	CXCL2	**4,620918**	**7,779355**	**5,982815**	**16,28218**
5820400	CYP4B1	1,555315	**3,002428**	2,910063	1,606337
2760433	DCLK1	1,043228	**0,2904194**	1,22293	1,198733
5130692	DDX17	0,8253888	1,068829	**0,3186888**	0,8688548
6180348	DIO3	**3,255445**	0,9034575	0,872212	1,048731

5270736	DNHL1	1,032544	**0,3162436**	1,095145	1,281718
6960687	DUSP2	**4,098994**	**3,685294**	1,951786	**4,126336**
2450390	FASTK	0,8113817	**3,082221**	0,5830431	1,27944
450114	FBXL5	1,007445	0,8439706	0,9794412	**3,667974**
5290541	FBXO9	1,0641	1,65938	**0,322459**	0,5575637
5910332	FIGNL2	**0,2734522**	0,9313356	0,6085634	0,9336877
2260189	FLJ14166	1,300859	1,737328	1,039193	**3,016098**
6840066	FLJ45337	1,289817	**0,2702013**	1,405934	0,8418458
6100730	FLJ46552	1,75497	**3,071846**	1,242602	1,903013
4280017	FOS	1,155671	1,948068	**4,056836**	2,645858
1340167	FOXP3	1,087768	**3,887042**	1,562027	1,549136
5420373	FUT6	0,4951778	2,664658	**0,2749111**	1,347374
6130692	GABRB1	**3,173761**	0,891439	1,401758	0,6276259
3840400	GMPR	**0,2603495**	1,002326	1,272435	1,080257
5340017	H19	0,7722384	0,8252725	1,171104	**0,2357198**
730672	HGD	1,185825	**3,012566**	1,007436	1,169382
7000725	HS.132394	0,9663341	**3,494347**	1,487267	1,986334
1260070	HS.150147	1,056058	**3,416581**	0,6516493	1,525244
6900768	HS.162932	1,036651	2,090227	**0,3029531**	1,920155
3360170	HS.163813	0,4357976	**3,574491**	0,9439649	0,9697723
2650048	HS.253267	0,585202	0,8284618	0,8091374	**3,003312**
6900519	HS.407822	1,248474	**3,07575**	1,033609	0,7001643
6900082	HS.421200	0,4831148	**3,881632**	1,626693	1,039435
730681	HS.47141	**3,177186**	0,5033462	0,4295464	1,342316
3390050	HS.484967	1,730272	**0,214378**	1,139803	1,948992
3870368	HS.527174	**0,3189452**	1,594399	0,8994039	0,9087678
5260167	HS.539177	1,887052	**3,128604**	1,435668	0,8840304
5290497	HS.540111	0,6385243	0,7712972	**3,01015**	1,771929
5700326	HS.542265	**3,929579**	0,9839553	0,9207538	0,5263394
2480491	HS.543097	0,9233336	**3,403158**	1,750232	0,8288471
4880241	HS.544914	1,392447	1,241152	0,8105588	**0,2967646**
1660437	HS.545589	0,3855146	2,667706	2,118108	**3,531147**
1570132	HS.549668	**3,505239**	0,4377305	0,6481511	1,087672
5910129	HS.549823	**0,2952034**	1,049047	1,235165	0,3756528
130195	HS.561604	0,5348192	0,6913961	0,6547982	**3,203995**

5960086	HS.562219	0,5246498	2,632425	1,267735	**3,464096**
2190041	HS.98462	1,543113	**3,06934**	0,8111399	1,272528
4040576	IL6	**3,856587**	2,778092	1,485888	2,599246
1570553	IL8	**5,964862**	**7,97365**	1,73849	**7,188703**
1980309	IL8	**4,62512**	**4,992615**	1,940345	**8,370797**
1820332	INSIG1	2,111597	1,73911	**3,668454**	**10,20621**
4040474	INSM1	1,110001	**3,40123**	0,8751904	0,7176682
7550500	JUNB	2,394651	**3,100962**	1,719416	2,116379
1470053	KIAA1244	**3,378979**	0,9883894	1,011196	1,359532
3990544	KIAA1666	0,4725696	**7,916166**	**6,821172**	**5,347475**
2100746	KIAA2010	2,706278	**3,307096**	1,85867	0,9801149
6290673	KLC2	0,9172528	**0,3009035**	0,9658512	0,8938444
5720184	KLF10	0,5998645	0,6256972	**0,1761282**	0,6852531
2350348	KRTAP1-3	**3,145697**	**6,660139**	0,5666649	1,369058
290446	LOC100128074	1,279815	**3,190472**	1,070882	0,7841643
6590563	LOC100129138	1,565138	**4,834883**	1,077641	1,40652
380487	LOC100129466	1,067975	1,136147	1,396075	**3,081289**
460189	LOC100130776	0,433785	**5,026352**	2,182864	2,397596
3460044	LOC100131193	0,7702207	**3,072215**	1,160671	1,286577
6200050	LOC100133554	0,8787	1,801833	1,078873	**3,3099**
4180180	LOC100133950	**0,2970659**	1,457816	0,6504056	0,5211301
3990386	LOC100134209	0,7299337	**3,804224**	0,6781151	1,147227
5220136	LOC220930	0,939723	**3,695652**	1,474746	0,9559682
730608	LOC285550	0,919295	**0,2764829**	1,329847	0,9549852
4250450	LOC388237	0,9963457	0,492785	**0,289328**	**0,2645709**
5690669	LOC389517	0,8601767	**3,58513**	1,460053	1,633067
6220364	LOC401629	**3,148228**	0,9782096	0,9793888	0,7757782
3440382	LOC402538	**0,303955**	0,9894025	0,9632904	1,246243
5090164	LOC440350	0,8199775	0,8551648	**0,2425306**	0,5134134
3190139	LOC440792	**3,505213**	0,9250695	0,9631301	1,064347
1850750	LOC441782	**0,315976**	1,973524	1,873454	1,349176
7330209	LOC572558	**4,317574**	2,382854	0,7644885	0,570591
2680286	LOC642425	**3,418268**	0,9088636	1,25921	1,021318
7150692	LOC642732	1,202422	**4,862957**	0,6167388	1,040789
1850044	LOC644065	1,096999	**3,052621**	0,9571976	1,127522

4850520	LOC644093	0,8446301	**3,354093**	1,243094	0,8621977
520093	LOC644338	0,94107	0,6421888	1,593361	**3,703692**
6270433	LOC644672	1,768673	**3,746936**	1,080105	0,9723387
4830288	LOC644701	**3,091007**	0,9645354	1,064836	0,7820551
6580209	LOC646208	1,09969	**3,329731**	1,039576	0,5828499
450224	LOC646439	**0,3157611**	0,6372947	0,7208813	0,9849367
1410327	LOC646496	1,549229	**3,691815**	1,130738	0,6519555
6020703	LOC647086	**4,303861**	0,4911491	1,658619	0,5787844
2190692	LOC647306	2,262169	**3,105164**	0,4967707	0,6822935
770487	LOC648581	1,517159	**0,3040158**	0,9336125	1,688581
4150338	LOC648815	**3,189015**	0,9980452	1,223642	1,198943
1260121	LOC648963	**3,321424**	2,154319	1,60322	0,469478
4250070	LOC649169	0,5003455	**3,175157**	0,6878486	0,9953658
6550274	LOC649679	0,6291462	0,9219107	1,26181	**3,229518**
5550520	LOC650628	1,942014	**4,43419**	0,8301249	1,206239
2190053	LOC651008	0,7408177	**4,126105**	1,338931	1,077482
3610438	LOC651137	0,8615851	**3,034135**	1,328942	0,7144698
6280373	LOC652787	**3,279812**	1,042797	1,622075	0,9860455
5220279	LOC723972	0,9443183	**3,001576**	0,6870557	1,509938
6520575	LOC728255	1,724844	**4,090688**	0,8954111	0,9764705
3940673	LOC728285	1,424541	**3,219892**	1,217175	2,139649
2190551	LOC728602	0,7860347	**3,181158**	1,712244	0,6675273
6770632	LOC728802	0,7194986	**3,017026**	1,066714	0,9796501
3060544	LOC728919	1,01434	**3,200629**	1,506987	2,011089
60338	LOC728946	0,822989	**4,620793**	0,5675926	1,062232
2650242	LOC728951	1,804907	**4,628035**	0,8669198	2,096613
770653	LOC728956	1,716566	**3,674238**	0,9980767	1,306756
6280452	LOC729324	**4,446665**	1,706042	0,6151472	0,9619201
580487	LOC729486	**4,190594**	0,9607814	1,015263	0,795423
2340437	LOC729859	**0,329382**	1,88096	1,893259	0,7966901
1510520	LOC730020	1,430012	**0,3196297**	0,8455133	1,04567
730709	LOC730525	1,625755	**3,651265**	0,9678959	0,9315074
4150446	LTBP1	2,410202	0,9927697	**0,2564085**	1,241527
5490367	MAGEA6	0,9397301	**0,2874547**	0,7614214	0,9482679
1470215	MAP3K8	**4,351634**	**3,086638**	2,912537	**6,747578**

20292	MCOLN3	**0,2971805**	1,695767	0,8221219	0,5315114
2600553	MCRS1	1,067103	1,849066	**0,3012887**	0,9555935
990070	MEST	0,8060015	2,590759	**0,3227827**	0,5146533
5910546	MGC4677	**3,42813**	**3,194242**	1,143797	1,414922
2260349	MIR1974	0,6564263	**5,990511**	1,51788	**3,432609**
3990307	MIR302C	**3,407011**	**3,546338**	1,987756	1,880198
4480685	MIR886	0,3911659	**3,478786**	2,241591	2,163694
780102	MLL3	0,7427763	**3,150197**	0,8358844	1,313689
3120392	NCDN	0,616663	0,9252807	1,288184	**0,3180796**
1170450	NGEF	1,061883	**0,2620055**	1,022206	0,6623794
630253	NOC4L	1,29571	**3,222599**	0,5220686	0,6097632
5260204	NR4A1	2,921181	**5,12263**	0,7615168	1,047035
6200026	NR4A3	1,168043	**4,553829**	**4,089621**	1,484958
990768	OAS3	0,9866416	**0,3044831**	1,28196	0,7684552
4390615	OPN3	0,5645198	**3,482884**	0,7835923	1,079808
1440484	PATE1	0,8329248	**3,466967**	0,9987844	0,9531384
620338	PBRM1	**3,215736**	0,737515	0,8100752	1,282548
380523	PDLIM5	1,694909	**3,371935**	0,4893207	1,625444
6280097	PLCB4	1,271791	1,389022	0,7250945	**3,091684**
2750367	PMAIP1	1,067694	1,163461	**3,099714**	1,361291
2140189	PSMD9	0,8972656	**0,3243912**	0,9327319	0,7221833
2260477	PTGS2	**6,112797**	**5,161868**	**4,32187**	**6,152825**
1820632	PTGS2	**4,382747**	**3,57512**	**3,048587**	**4,107662**
2480681	RBM4	1,596534	**3,263705**	0,7876263	1,068122
620239	RCAN1	**3,590458**	**3,538348**	0,8560772	2,656061
10026	RMRP	0,4934396	**6,829357**	**3,809812**	1,235366
2640719	RN7SK	**0,3257661**	**3,686631**	**5,855864**	**4,278602**
990176	RN7SK	**0,2032487**	**4,364679**	**6,948196**	**7,808771**
5690523	RNASEH2C	0,7390152	**0,3083463**	1,144977	0,5712836
5670152	RNU1-3	1,334595	**4,029978**	2,105802	2,980356
2970482	RNU1-5	1,166446	**3,681452**	2,067749	2,467642
7330681	RNU1A3	1,311332	**3,25197**	1,828278	2,253531
5670753	RNU1F1	1,323239	**3,195265**	1,970035	1,974419
2360091	RNU1G2	1,384125	**3,71403**	2,117392	2,834196
6960048	RNU6ATAC	0,8928777	**4,468182**	2,589964	**3,30403**

2750050	RNY5	**0,2157045**	1,912896	1,097424	1,832509
840242	RPL13	1,193544	2,492211	**0,329664**	1,018224
3450753	RXRG	0,8048805	**3,33211**	0,750378	1,168399
1240093	S100A14	0,9869198	**3,177992**	0,3392214	1,502837
3420037	SELS	**0,2979728**	0,5658749	0,5896825	1,197466
5810095	SERPINB2	2,774786	**4,799103**	2,350577	**9,083377**
6350209	SERPINB2	**3,418744**	**3,422387**	2,005572	**5,302586**
6940553	SEZ6	0,7699443	**0,2967636**	0,845663	0,6396
5310048	SF3B1	**0,3242218**	0,8183196	0,4594126	0,9922867
7650523	SHISA2	**3,223353**	2,332115	2,074546	**3,021754**
830022	SLC12A2	**0,3042542**	1,113481	1,165075	**3,226976**
3890402	SLC25A24	**6,411805**	**11,16116**	2,070489	**4,00352**
5490601	SLC40A1	**4,151508**	0,8719032	0,8984643	1,207582
4230477	SNORA76	**0,311672**	1,081326	1,301558	1,469241
2510164	SNORD3A	0,9092273	1,85751	**3,542389**	0,9074144
580161	SNORD3C	1,127802	1,745995	**4,088701**	1,006155
380685	SNORD3D	0,8007043	1,597259	**4,915395**	0,820849
4810671	SPTLC1	0,8483723	**3,064003**	0,555908	1,237349
2070440	ST8SIA4	0,8683283	0,5957634	**3,244537**	1,284885
290332	TAF13	0,4575483	1,075326	2,243978	**3,340592**
3360326	TAGLN	1,244827	**3,421963**	0,5921015	1,883088
4280674	TFPI	0,7360882	**3,152501**	1,643533	2,432204
1990026	THSD1	0,5540726	**3,086588**	0,825916	0,895084
1470273	TMEM35	0,7962913	**3,07941**	0,6676417	0,9826267
4010039	TNNT2	1,509319	1,194726	1,084118	**0,2792105**
6450484	TRPS1	**3,835884**	1,065009	1,709407	1,137005
770494	TSC22D3	0,7669975	**3,02848**	0,7606265	1,39157
4120148	TTC39B	**3,109303**	1,198029	1,337745	1,403657
3930241	U2AF1	**0,2783099**	0,8580953	1,09174	0,9642395
7400372	WSB1	0,4495932	0,64665	**0,3008561**	0,7210644
5570689	XAGE1	1,476957	**3,185065**	1,744652	0,9300943
3610367	ZIM2	0,7345725	0,5544626	**3,356801**	0,6040263
3930253	ZNF12	0,6142385	**0,3257053**	0,5854767	0,5829732
4850270	ZNF264	1,482872	**13,1416**	0,8718864	**3,813202**
6560594	ZNF322B	1,057274	**3,17542**	0,8345412	0,5430454

2100086	ZNF568	**3,722507**	0,7705103	1,420136	1,022752
5490402	ZNF580	1,15265	1,328641	1,575234	**3,298034**
4010110	ZP3	**3,781727**	1,324944	0,682661	1,875506

Chapter 5 : Materials and Methods Complement

Before proceeding to a general discussion of the material presented thus far, we furnish a brief complement to the account of a few of the more involved techniques and protocols employed in the two scientific manuscripts we have just seen, whose description therein may have been slightly truncated owing to the requirements of scientific journals. The techniques and protocols chosen for explanation in greater depth below are those for which we felt that more detail might be necessary or helpful, and are intended to serve as supplemental reference for others wishing to reproduce or continue this work.

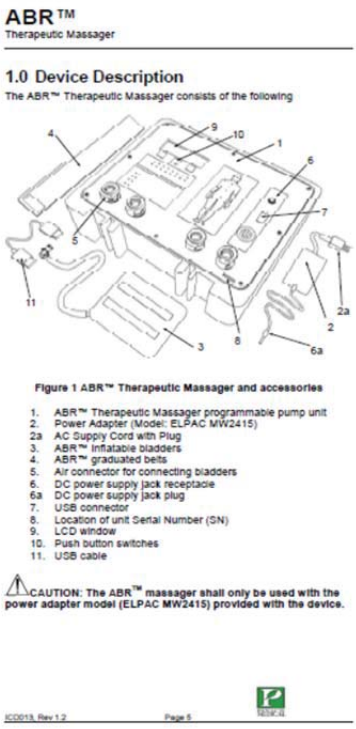
5.1 Use of the ABR Therapeutic Massager

5.1.1. Description of Device

To remind the reader, the ABR Therapeutic Massager device was adapted for our *in vivo* work in order to supply a desired mechanical stimulus pattern externally to the body of a test subject. This device has been certified by numerous health and regulatory agencies in North America, the EU, and around the world, including a Health Canada authorization for clinical use on patients. A picture of the tactile user interface of the machine is shown in **Figure 5.1**, while diagrams of the device (blow-up and operating interface) are found in **Figure 5.2**. Originally, its creators intended the device to relieve minor muscle aches and pains via the use of air-inflated bladders applied externally to the body.

Figure 5.1 View of ABR Therapeutic Massager Interface.

Figure 5.2 ABR Therapeutic Massager blow-up (left) and interface (right) device diagrams.



ABR™
Therapeutic Massager

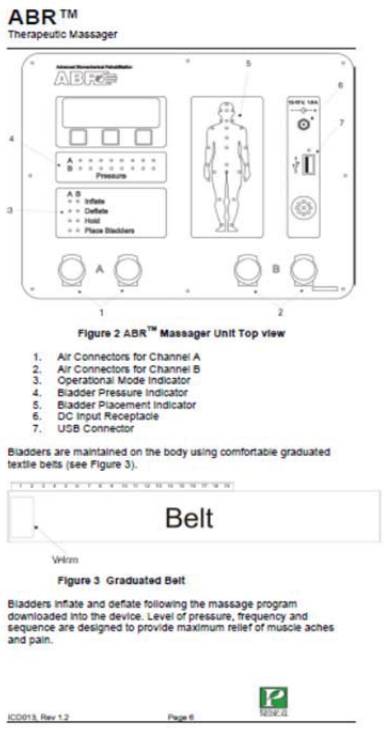
1.0 Device Description

The ABR™ Therapeutic Massager consists of the following

Figure 1 ABR™ Therapeutic Massager and accessories

1. ABR™ Therapeutic Massager programmable pump unit
2. Power Adapter (Model: ELPAC MW2415)
2a AC Supply Cord with Plug
3. ABR™ inflatable bladders
4. ABR™ graduated belts
5. Air connector for connecting bladders
6. DC power supply jack receptacle
6a DC power supply jack plug
7. USB connector
8. Location of unit Serial Number (SN)
9. LCD window
10. Push button switches
11. USB cable

⚠CAUTION: The ABR™ massager shall only be used with the power adapter model (ELPAC MW2415) provided with the device.



ABR™
Therapeutic Massager

Figure 2 ABR™ Massager Unit Top view

1. Air Connectors for Channel A
2. Air Connectors for Channel B
3. Operational Mode Indicator
4. Bladder Pressure Indicator
5. Bladder Placement Indicator
6. DC Input Receptacle
7. USB Connector

Bladders are maintained on the body using comfortable graduated textile belts (see Figure 3).

Figure 3 Graduated Belt

Bladders inflate and deflate following the massage program downloaded into the device. Level of pressure, frequency and sequence are designed to provide maximum relief of muscle aches and pain.

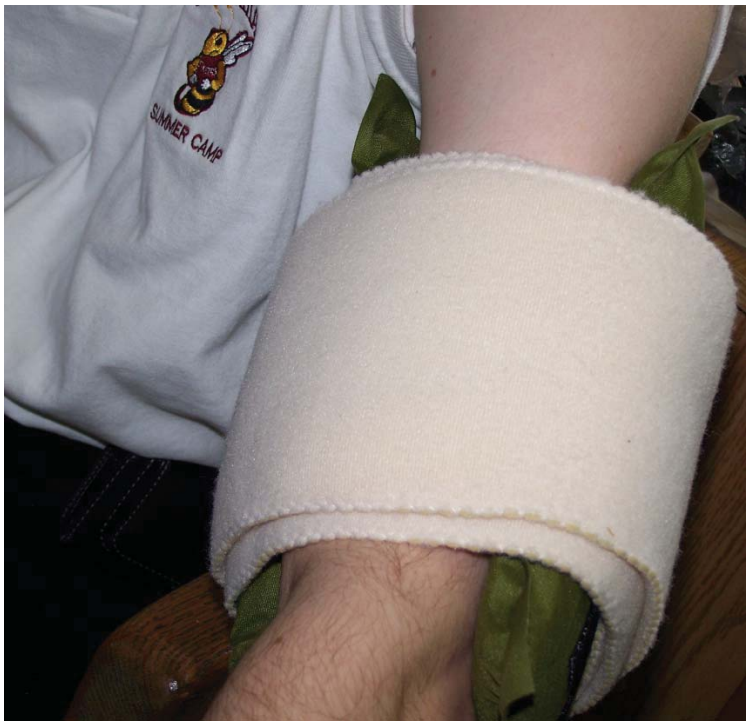
The programmable pump unit is electrically powered and activates a series of pumps which inflate and deflate the bladders according to the program downloaded to the device. As can be seen in **Figure 5.2** on the right, two channels are available to connect bladders, channels A and B. Each channel can accommodate one or two bladders. The massager program itself cannot be programmed beyond certain set limits of massage time and pressure. The programmable nature of the device is comparable to other programmable massage devices that allow a user to select from a pre-defined set of massage programs.

Massager bladders come in several sizes and dimensions which can be chosen to best suit convenient use on particular areas of the body. Each bladder connects to the pump unit using a latex-free hose. The medium-sized bladders used for the work presented in Chapter 3 are shown with hoses attached in **Figure 5.3**. Bladders are maintained on the body using comfortable belts secured by Velcro. The belts are designed to be worn over clothing, although we preferred to minimize such separation between bladders and skin wherever possible. Air-pressure inflation of the laminated bladder assembly supplies the actual massage force to the body. The bladder assembly contains a foam core that can only expand to a certain thickness at the desired pressure, at which point further inflation pressure ceases to result in expansion due to the presence of an air-valve that will bleed out the excess.

The massager device is reprogrammable with the help of the manufacturer's software, obtainable upon special request. Although the frequency of inflation/deflation cycles cannot be greatly modified, the magnitude and duration of mechanical stimulation can be varied from defaults, and programs may be generated to store customized parameters and user-specified stimulation patterns. A program need only be created and stored to memory once before use in protocols.

Figure 5.3 View of medium-sized air bladders, with hoses attached. These bladders were used in the work presented in Chapter 3.

Figure 5.4 Final placement of air bladders on test subjects, secured with Velcro belt.



5.1.2. Guidelines and Precautions

For our purposes, as there was no preset program that met our needs, we elected to create a custom program to supply the desired stimulation waveform used in our *in vivo* work, a sinusoid varying from 0-4 psi, at 0.006 Hz for 90 minutes. Performing our stimulation protocol then is simple and merely entails the following steps:

1. Ensure that the test subject is comfortably installed, relaxed, and lying flat on his/her back on the bed, with a pillow to support the head, if desired.
2. Connect the AC adapter and power on the machine.
3. While the operating system loads, connect the air hoses to the bladders you wish to use and plug the pair of opposite ends into either Channel A or Channel B of the device. During this time, assuming that the test subject has been resting for at least 5 minutes in position on the bed, the initial blood sample may be drawn.
4. From the tactile user interface, select the program to run, either a preset or one of the custom user-designed created via the manufacturer's software.
5. The device will now evacuate all air from the bladders automatically, after which they may be secured to the patient's body part of choice (for us, the arm, above the elbow) by placing them and wrapping the Velcro belt all the way around completely. In our case, to maximize the surface area of stimulation, we placed the two bladders on the arm by positioning them on opposite sides of the arm, with no overlap. **Figure 5.4** shows a visual of the final placement of the air bladders on the upper arm of a test subject, held in place with the aid of the Velcro belt, as specified before.
6. Once done, one may begin the stimulation program selected by pressing Start on the command interface of the device, and letting it run to completion. For our experiments, this meant of course a stimulation duration of 90 minutes, punctuated by blood drawings from the non-stimulated arm at 30 minute intervals as described before in Chapter 3.

As one can see, the protocol is not terribly complicated. However, there are a few precautions that we observed. First, we did not permit the patient to leave the bed for any reason (such as bathroom breaks) except in case of emergency, in order to maintain

continuity of stimulation. Also, we tried our best to minimize patient movement during the experimental protocol. We did not employ any kind of physical implements or restraints to restrict movement, as we felt this might be slightly drastic, particularly for a pediatric population. However, all subjects were advised verbally at the outset to keep still on their backs to the extent possible, with gentle reminders at occasional intervals when necessary. We allowed simple, quiet activities for the children such as reading or listening to music to help keep them entertained, so long as the activity did not encourage any superfluous or excess motion. These provisions were followed in order to assure, within reason, that as little loading or stimulation from sources other than the ABR Therapeutic Massager as feasible was introduced, thus helping to preserve experimental integrity.

5.1.3. Choice of Experimental Parameters for Mechanical Stimulation

Our chosen stimulation parameters, it must be stated, are in some sense arbitrary from a scientific point of view. Since this sort of work has never been tried before, one must simply pick a starting point and start optimization work from there. With so little useful guidance from foreknowledge to help design the protocol, we therefore considered the facts that a) an experimental protocol, not to mention a potentially useful (and marketable) future scoliosis test should not cause a patient/subject undue inconvenience, and so making and designing a test that forces a subject to sit in a clinic or wait in a hospital for hours on end is pointless, since even if such a time scheme was “successful”, i.e. was functional and yielded results, it just would not be useful in a practical clinical setting; and b) from a subject and study consideration point of view, one must remember that the target subjects are young adolescents, for whom restricting movements and keeping them focused and well-behaved for the duration of the protocol may prove more difficult the longer they are forced to stay. In fact, any recruitment of subjects at all might be troublesome if the protocol were much longer than what we designed. Weighing all of these considerations, we felt that the design of protocol for 90 minutes of application struck a good balance between scientific value and progress with useful data towards development of a scoliosis test, patient/subject convenience, and feasibility of carrying out the study. In terms of location of application, the arm was chosen for its convenience of application (a good and simple characteristic to have for a future test) and its distance

from the spinal musculature (recall that we hypothesized that forces *away* from the spine were capable of raising OPN levels). Again, we have simply chosen an anatomical location here in lieu of knowledge of any superior option.

As regards the magnitude of pressure, we chose to employ the machine's stated maximum capacity of 4.0 psi simply because we wished to supply a reasonable load that we believed was likely to be strong enough to elicit a response, if there was one to be had, while still being safe for the subject. Now, is up to 4.0 psi in fact a safe pressure to apply to the body for extended periods? One may be concerned about this issue; however, it must be pointed out that a force of about 1.0 psi is already normally present on persons who cannot leave their bed or wheel chair due to motor function or other problems [358]. Moreover, references can be made to the pressures normally present on the body during actions as mundane as sitting or standing. In particular for an average adult male (evaluated by rudimentary calculation of subject weight/area):

Typical pressure on Buttocks Sitting: ~ 2.3-2.9 psi [359]

Ordinary pressure on Feet Standing: ~ 6.7-10 psi [360, 361]

Additional reference can be made to the average human systolic blood pressure, as exceeding this pressure could cause localized discomfort due to lack of circulation.

Average Systolic Blood Pressure: ~ 2.32-2.5 psi [362]

In addition, during blood pressure measurements, it is common practice for the inflatable bladder placed around the arm, leg or finger to be inflated well beyond the systolic pressure to ensure an accurate reading. This inflation is usually done for a longer period of time than our bladders during a massage session.

Average pressure applied by sphygmomanometer: ~ 3.3-5.8 psi [363, 364]

It can be seen from the above data that our choice of pressure to be supplied from the ABR™ Therapeutic Massager was well within the orders of magnitude of pressure normally borne by the human body without any trouble whatsoever.

Figure 5.5 Front view of Harvard Apparatus PhD Ultra Syringe Pump.

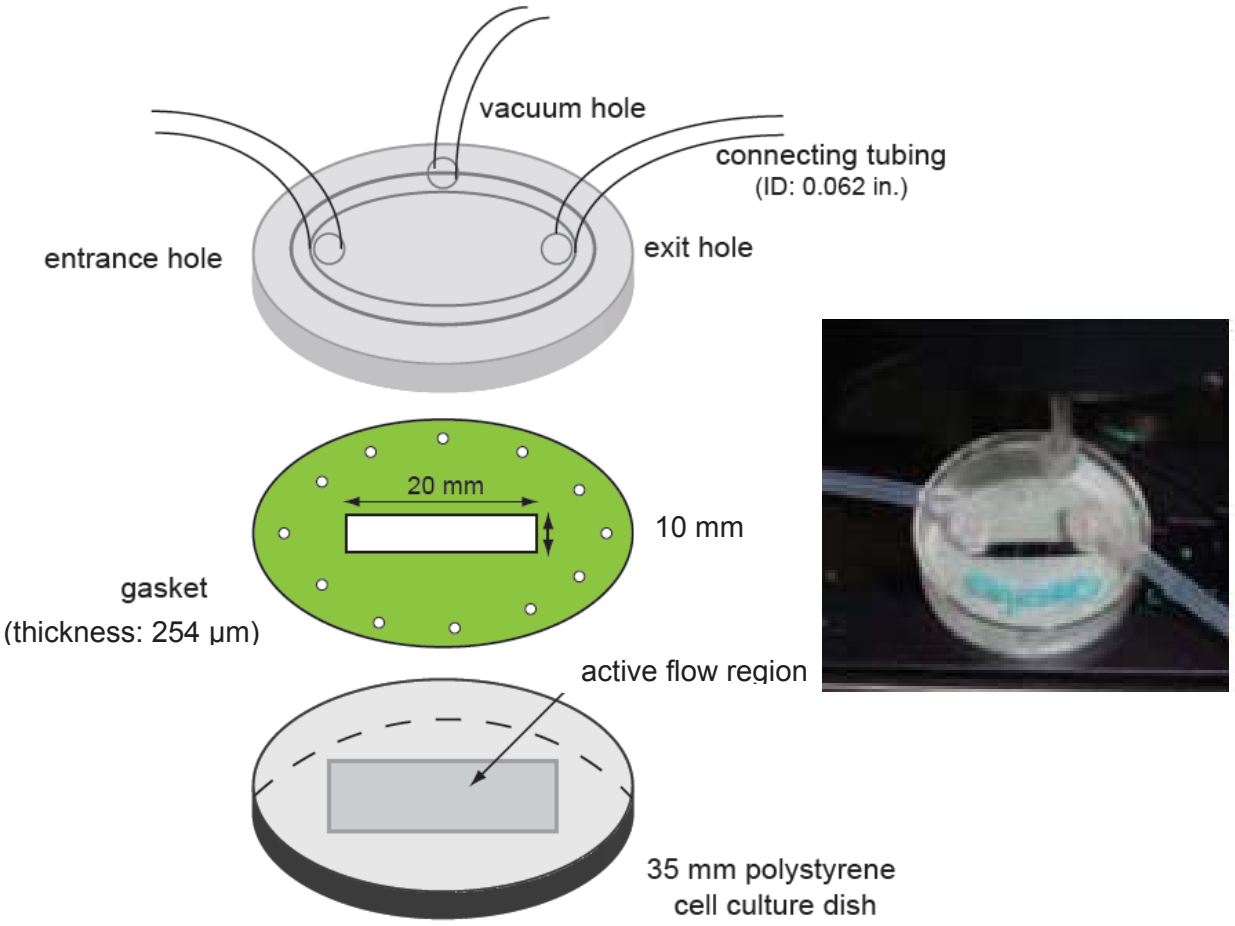


Figure 5.6 Parallel plate flow chamber schematic (left) and photograph in operation (right).

5.2 Parallel Plate Flow Chamber Precautions

As we have just seen, the parallel plate flow chamber setup played a huge role in our *in vitro* work, permitting us to apply fluid shear stress to cells in a predictable way. Construction of this setup consists principally of setting up and programming the Harvard Apparatus PhD Ultra Syringe Pump (**Figure 5.5**) correctly, and then observing the appropriate steps and precautions when physically assembling the Glycotech flow deck with the experimental samples contained in the fully confluent 35 mm cell culture dishes (shown schematically and in real-life photographic image in **Figure 5.6**) in preparation for fluid shear stress application.

5.2.1. PhD Ultra Syringe Pump Setup

The first step is to design a program (an "Operating Method") in the pump software with the proper parameters to give the desired pattern of fluid shear stress. Thus such a program will define:

- Details about the syringe being used for the application
- How rapidly to infuse or withdraw the target fluids
- A target volume or target time for the dispensing or withdrawing operation
- The pumping pattern you want to employ (e.g. pump at a continuous rate, in a ramped fashion, using variable rate steps, etc.)

In this particular project, the pump needed to produce a specific waveform of applied force to implement (sinusoid of amplitude 2 Pa, frequency 0.5 Hz, for 90 minutes). As a representative guide to programming the pump, we will now describe the creation of the specific Operating Method employed in our work.

When the PhD Ultra Syringe Pump powers on, the touchscreen user interface will appear on the display as in **Figure 5.7**. From this screen, we must first create an Operating Method. This takes the following steps:

1. Touch the button 'Method Select', and then 'New Method' on the subsequent screen.
2. On the Method Name screen, type the name of your Method using the onscreen keyboard, and press the 'Accept' button (check mark) when finished.

3. To begin creating one's desired pattern of fluid infusion/withdrawal, choose the 'Step Definition' button to enter the Method Steps Summary screen.
4. Choose the 'Add Step' button to access the Method Steps Menu screen. The available options include: Constant Rate, Ramp, Stepped, Pulse, Bolus, Concentration, Autofill, and Advanced Options. For the purposes of our own work, a two-step method comprising a short Bolus infusion followed by an Autofill profile was all that was required. Naturally, more complex methods can be created to suit different purposes, if necessary.
5. For the Bolus infusion, we set the target volume to 2.5 mL at a flow rate of 18 mL/min. When in operation, this infused volume saturates the entirety of the flow tubing system and flow deck with fluid prior to commencement of the actual desired experimental flow scheme, which will have the same magnitude of flow rate.
6. For the Autofill step which defined our experimental flow pattern, we selected an Infuse/Withdraw mode, choosing a flow rate of 18 mL/min in each stage. This flow rate was calculated as in the manuscript of Chapter 4, in order to give a sinusoidal shear stress waveform varying between ±2 Pa. The Volume per Cycle was defined to be 300 µL, providing the 0.5 Hz frequency at the given flow rate. The Total Volume may be set to any sufficiently large figure so long as the time taken to pump that volume is longer than the desired period of stimulation. The use of this setting was designed to be a method of controlling and measuring the duration of stimulation; we, however, found it to be unreliable and inaccurate, so we simply input a large arbitrary volume (2 L) to ensure a long period of flow, and measured the 90 minutes dictated by our protocol with a conventional stopwatch.
7. Once these steps are completed, save the method by touching the 'Accept' button.

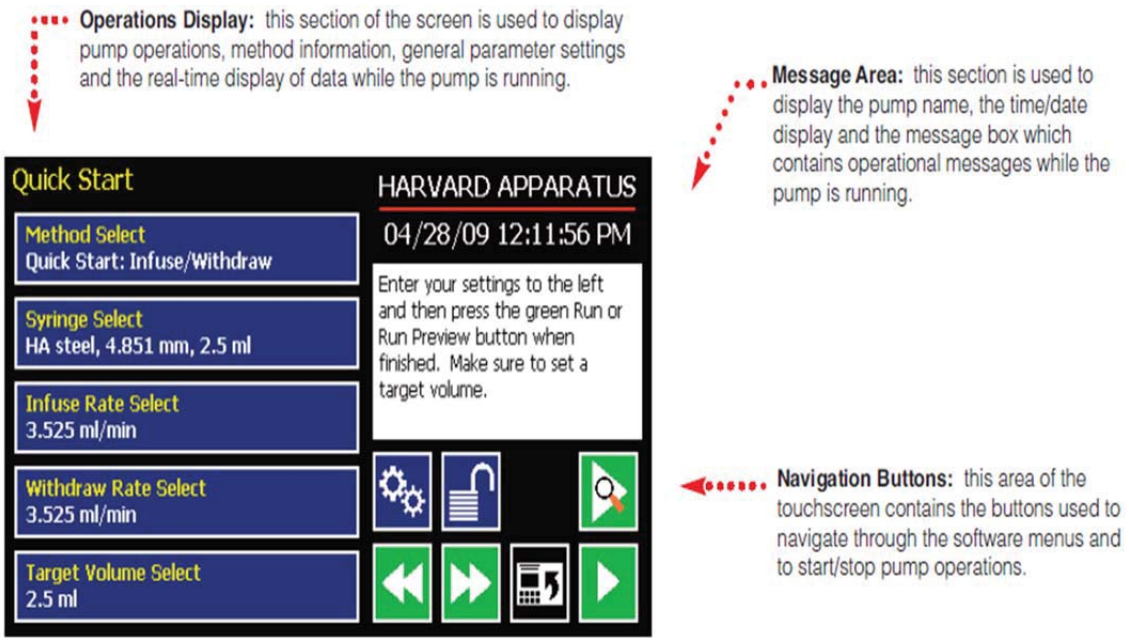


Figure 5.7 User display interface of Harvard Apparatus PhD Ultra Syringe Pump.

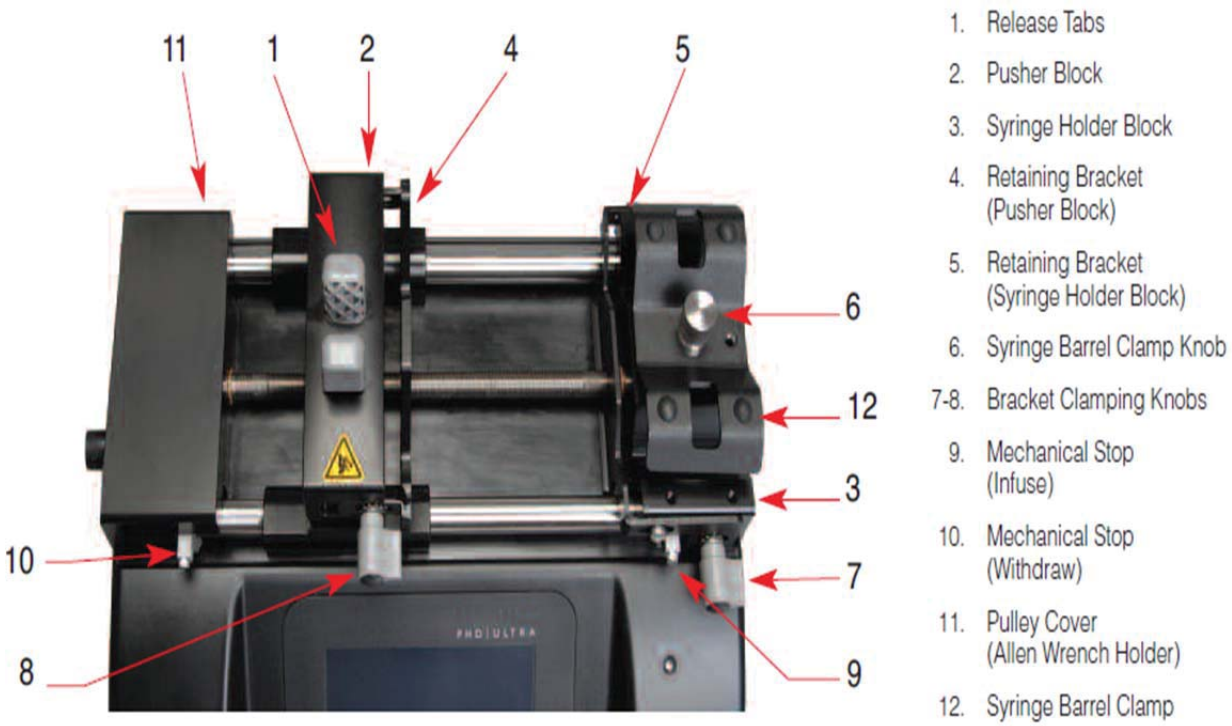


Figure 5.8 Top view of PhD Ultra pump showing useful controls for syringe loading.

5.2.2. Preparation for Fluid Shear Stress Application

Readying the samples for subjection to flow or no-flow conditions on experiment day is not difficult, though it does require a fair amount of lead time. Before even removing any samples from incubation, it is necessary to prepare and properly clean and disinfect the workspace (cell culture hood) as well as the Glycotech flow deck and tubing. The culture hood is most efficiently decontaminated with UV light; however, one must ensure that the Glycotech flow deck is *never* exposed to it, as this is certain to cause premature degradation. Instead, we cleaned and wiped down the flow deck using nonabrasive tissue paper before and after every use with 70 % ethanol solution, as well as RNAse Away (Invitrogen, Burlington, ON).

Assuming the precautions above were observed, loading samples into the parallel plate flow chamber setup for stimulation takes the following steps:

1. Remove the sample to be tested from cell culture incubation. Aspirate all culture media away, and add 1-2 mL of PBS 1X solution to the 35 mm dish. Gently rock the dish to evenly distribute the PBS, and then aspirate again completely.
2. Attach the rubber gasket of preferred flow field dimensions to the flow deck. Inverting the flow deck such that the now-attached rubber gasket faces upwards, pipette a small bolus (perhaps 15-20 µL) of fresh culture media onto the rectangular flow field. Keeping the deck inverted now, turn the 35 mm petri dish in Step 1 upside down and place the dish onto the deck. The bolus of fluid helps keep the two parts together.
3. Fill the syringe to be loaded onto the PhD Ultra Pump with fresh, warm culture media, and attach a Luer connector to the end. In this project, we used Beckman-Coulter 10 mL plastic syringes (Beckman-Coulter, Mississauga, Ontario) for all flow/no-flow experiments. Load the filled syringe onto the PhD Ultra Pump's syringe rack. **Figure 5.8** shows a diagram of some important controls for syringe loading that may prove helpful.
4. At this point, the loaded PhD Ultra Pump may be moved into the incubator maintained at 37 °C, 5% CO_2. Also, fill a sterile 15 mL capacity Falcon tube with 5-

10 mL of warm fresh media and place into a rack inside the incubator. This is the "overflow reservoir" of **Figure 4.2**.

5. Connect tubing from the vacuum pump to the vacuum port of the parallel plate flow deck, the inlet port to the Luer connector of the syringe loaded onto the PhD Ultra Pump, and the outlet port to the overflow reservoir. Switch on the vacuum pump to retain the parallel plate flow chamber.
6. Powering on the syringe pump, select the Method to run. Before commencing the run, it is necessary to specify to the software the syringe one has loaded. This is easily done by pressing 'Syringe Select' and selecting the proper syringe type, size, and manufacturer. At last, the method may now finally be executed; press the 'Start' button to begin, and wait for completion.

5.3 qRT-PCR Primer Design Strategy and Guidelines

Optimization of primer design is critical to ensuring quality of results in subsequent qRT-PCR experiments. In this work, we aimed for high standards of rigour in the process of selecting them. From NCBI's Primer Blast utility, the following parameters were input for screening purposes:

- PCR Product size: 80-250 bp
- Number of primers to return: 10
- Primer melting temperatures: 54-56° C, optimum 55 °C
- Primer must not span an exon-exon junction
- Primer is allowed to amplify mRNA splice variants
- Primer size: 18-22 bp, optimum 20 bp
- Primer GC% Content: 50-60%
- Concentration of divalent ions: 1.5 mM
- Concentration of dNTPs: 0.2 mM

Other specifiable parameters not mentioned above remained at default values.

If a primer pair was deemed promising after the preceding screening, the DNA product that would be created was further checked in mFold, a web-based utility, in order to identify through thermodynamic analysis any potential for formation of undesirable

secondary structures (such as loops or hairpins) during annealing. To perform this check, the following parameters were input:

- Annealing temperature: 55° C
- $[Na^{+}]$: 1.5 mM
- $[Mg^{2+}]$: 0.2 mM

Again, unless otherwise specified, other parameters not mentioned above remained unchanged from default values. After the analysis run, any primer pair demonstrating energetically favourable formation (enthalpy change $\Delta G \leq -1$ kJ) of unwanted secondary structures was rejected.

5.4 Hierarchical Clustering of Microarray Data

Hierarchical clustering can be a powerful method of illuminating inherent relationships in large sets of data, such as those obtained through microarray experiments. In such bioinformatics analyses, its main utility is to construct groups of co-expressed genes. The basic idea is to assemble a set of genes into a tree, where they are joined by very short branches if they are very similar to each other, and by increasingly longer branches as their similarity decreases. However, it is only capable of doing so usefully if an appropriate algorithm is chosen and justifiable for the particular problem at hand.

Starting from a theoretical perspective, we recall that the first step in hierarchical clustering is to calculate the distance matrix between the gene expression data. Once this matrix of distances is computed, clustering may begin. Cluster 3.0, the software used in our work for clustering analysis, relies on agglomerative hierarchical processing, which consists of repeated cycles where the two closest remaining items (those with the smallest distance) are joined by a node/branch of a tree, with the length of the branch set to the distance between the joined items [365]. The two joined items are removed from list of items being processed replaced by an item that represents the new branch. The distances between this new item and all other remaining items are computed, and the process is repeated until only one item remains. Note that once clustering commences, both items that are true items (e.g. a single gene) and items that are pseudo-items

containing a number of true items are treated. There are a variety of ways to compute distances when we are dealing with pseudo-items: centroid linkage, single linkage, complete linkage, and average linkage [366].

We happened to select and use an unweighted (i.e. all gene observations are treated equally) hierarchical clustering algorithm using centroid linkage as the clustering method and Euclidean distance as the similarity metric. Under centroid linkage, a vector is assigned to each pseudo-item, and this vector is used to compute the distances between this pseudo-item and all remaining items or pseudo-items using the same similarity metric that was used to calculate the initial similarity matrix. The assigned vector is the average of the vectors of all actual items (e.g. genes) contained within the pseudo-item. Computing the similarity, or dissimilarity, between vectors using Euclidean distance involves simple calculation of the L_2 norm of vectors [367]. This similarity measure is perhaps the most commonly used in microarray data clustering and analysis, along with Pearson correlation [368].

It is important to note that there is no “best” clustering method that is manifestly superior to all others; all possess inherent advantages and disadvantages. Our choices were made mostly due to the relative simplicity in computation and concept, though we fully recognize that picking other clustering options may also have led to unearthing of interesting and no less potentially valid gene relationships.

Practically, when operating the Cluster 3.0 software, running an analysis algorithm is an elementary task. Much of the work really lies in correct preparation and formatting of the data file to upload. One simply copies the ensemble of probe expression values of the genes to be clustered (calculated by application of the robust multiarray algorithm in FlexArray, as before) for all microarrays under consideration into a tab-delimited text file organized into columns, one column per microarray set of gene expression values. A column is inserted on the left to contain the gene names, and a row is inserted at the top to store the identifier of each microarray studied. No further processing of data is necessary; simply select the appropriate options and checkboxes to define the clustering analysis algorithm to be performed, and execute.

Chapter 6 : General Discussion and Conclusion

6.1 General Discussion

The present study investigated, for the first time, two key elements neglected heretofore in research on mechanotransduction in AIS: a) global *in vivo* mechanotransductive effects stemming from non-spinal forces; and b) fluid shear stress effects induced by mechanical loading on musculoskeletal cells. The recruitment of patients to undergo the novel protocol of mechanical stimulation as outlined in the first manuscript allowed a comparison of average changes of circulating OPN levels between healthy individuals and subject groups of different scoliosis severities as a result of non-spinal applied force.

To recapitulate what we have just seen through presentation of the preceding two scientific manuscripts, we discern the following key results in summary form:

- OPN was universally responsive *in vivo* to dynamic mechanical stimulation applied away from the spinal region, increasing across all severity classes recruited after loading. However, sCD44 was not found to be so.
- The change of circulating OPN levels measured *in vivo* in response to our mechanical stimulation was statistically significantly correlated to status of spinal deformity severity, with more severely affected subjects demonstrating lower magnitudes of ΔOPN.
- The aforementioned relationship was also dependent with age and gender, especially gender. Females tended to show lower magnitudes of change in circulating OPN levels as opposed to males, as did older subjects with respect to their younger counterparts.
- *In vitro*, fluid shear stress is a potent stimulator of gene expression change in osteoblasts from both normal and severely affected AIS subjects, with many genes (210) showing significant differential expression in at least one of the four experimental groups (controls and groups I, II, and III), and this pool from which we managed to identify two biologically interesting clusters for further study, with potential implications for AIS.

- However, in response to our simulated mechanical loading-induced fluid shear stress, only a very small number of genes are either mutually differentially expressed or statistically unchanged across all functional scoliotic subgroups while having the opposite condition in the control group. This indicates a great degree of difference in terms of mechanotransductive response as compared internally between AIS functional subgroups, as well as between control and AIS patients.
- COX2 expression was strongly up-regulated by OFF in all four *in vitro* experimental subgroups according to both qRT-PCR and microarray analyses; though qRT-PCR analysis showed very much higher up-regulation in Groups II and III than did microarray analysis for these groups.
- According to microarray analysis, PITX1 was strongly down-regulated by OFF in Groups I and III, though only meeting the significance p-value criterion in Group III.
- According to qRT-PCR analysis, OPN was strongly up-regulated by OFF in Groups II and III, but was unchanged in controls and Group I.

Now in this final section, I propose to develop an extended discussion of these and other aspects of interest encountered over the course of this project that have not already been discussed at length elsewhere, as well as some indications of where future research may be profitably directed.

Concerning the first three points relating to the *in vivo* work presented in Chapter 3, with the exception of the inverse correlation between curve severity and ΔOPN already commented upon in that section, most of these observations could have been expected given our previous experiences. For instance, the fact that OPN response *in vivo* was connected with age and gender was quite foreseeable. In our laboratory's previous study demonstrating the association of OPN with AIS, we had also seen a connection, observing that OPN levels were higher in younger children and in females [97]. In addition, since sCD44 has not generally been associated as yet with mechanotransduction as stated in the introduction, it should be no surprise to find it to be insignificant here too.

The ramifications and potential of our *in vivo* findings, at least from a clinical/diagnostic point of view, were already enumerated at some length in Chapter 3.

However, our *in vivo* results carry significance from a fundamental standpoint too, as they suggest, for the first time to our knowledge, that mechanical force and stimuli taken from one region on the human body (in our case, the arms) are capable of modulating factors (OPN) on a global level, factors that have known associations and effects on other regions of the body (spine) that may be quite some distance away, anatomically speaking. We have already seen in the introduction how much of the scientific focus to date concerning mechanical force as it pertains to AIS has been concerned with those forces in and around the spinal column itself. Now, with the advent of these last results, the way may be open for something of a paradigm shift on the subject of mechanical force in AIS, forcing both basic researchers and clinicians alike to take a wider-ranging view of this factor in their respective work.

For the fluid shear stress work presented in Chapter 4 and summarized above, it must be noted that although the spirit and concrete content of our work on fluid shear stress remain largely faithful to what our vision of a completed oeuvre would be at the outset, there remain a few minor gaps that we feel must be clarified before submission to our target journal. In large part, these pertain to qRT-PCR features in the study. First, we do recognize that the internal standard deviation of fold changes of some groups is quite large in the qRT-PCR verifications already performed upon the COX2, PITX1, and OPN genes. Thus, since the plausibility of much of the discussion presented in Chapter 4 about the consequences of our obtained fold change results for these three genes is predicated on the trustworthiness of the qRT-PCR values, we should certainly corroborate them through a repeat experiment, at very least for those group/gene combinations with substantial standard deviation. Further, it must also be recognized that these qRT-PCR results as presented in the second manuscript are of course incomplete without a full statistical analysis of the significance of the fold change values obtained, preferably a two-way ANOVA with experimental group and flow condition as factors. Lastly, we feel it would be desirable to enlarge the set of genes to be verified by qRT-PCR, perhaps encompassing all genes of a particular set of interest such as those that share common biological functions or differential expression patterns in certain clusters/functional groups. Ideally, this expansion would bring our total number of qRT-PCR verifications to

around 15-25 genes. Once these steps are completed, this should be more than sufficient to satisfy publication standards of a completed work for our target journal, JBMR.

Globally, this project's work may possess important implications, not merely in terms of basic theoretical knowledge of disease pathogenesis but also in applying practical clinical treatments. In speaking of either of these two aspects, biomechanical force is nearly always an implied element pervading the discussion. As an example, we have already seen that whether considering basic spinal asymmetries [184], column buckling [186], or modern theories of self-perpetuating spine imbalances (vicious-cycle) in AIS development and progression [177], biomechanical forces are always front and center in each of these theories. Yet the processing of such biomechanical force signals at the cellular/molecular level into chemical responses has hardly received any attention. In this project we have shown that this signal transduction may be enormously important to examine, as indications are that these mechanisms are significantly altered between normal subjects and AIS patients. On the clinical side, consider that throughout the historical evolution of scoliosis treatments, from the primitive Harrington rod implements [283] to modern dynamic bracing such as SpineCor [107] to drastic spinal fusions [267] to even physical exercises [369], in spite of the gross difference in era and method, the theme has invariably been the same: alter the mechanical environment of the spine in such a way that favours the arrest or even reversal of curve progression. The prevailing attitude, alas, is rather dogmatic in this regard, as these treatments tend to be applied according to the attending physician's clinical judgement, with the expectation that candidates meeting the set criteria for a particular treatment option, presumably all with comparable curve and physical characteristics, should then respond to treatments in a comparable manner [370]. This approach has been patently insufficient, as we observe from the literature that the positive effect of many of these treatments is still extremely controversial [371]. For example, researchers and clinicians alike are mystified when some report that the incidence of surgery is significantly reduced in brace-treated scoliotic populations compared with untreated groups [372-374], while others fail to achieve such outcomes [375]. This project adds a new dimension to the discussion, as with the coming to light of the evidence in the preceding two manuscripts, a possible reason begins to

emerge that these treatments may not always be working because of the variance in certain patients' basic mechanotransductive disposition. That is to say, due to the mechanotransductive distinctions between different scoliosis patients like we have just witnessed at both the global and cellular levels, *in vivo* and *in vitro*, respectively, one patient might simply process and respond to mechanical stimuli as in biomechanical force-based interventions like bracing differently than another, despite having similar curve and physical characteristics, leading to the reported discrepancy in effectiveness. Therefore, in years to come, the mechanotransductive profile of a patient could be another integral factor to weigh, clinically, when considering or designing treatment plans for affected persons.

6.2 Future Directions

As for future work serving to continue this project's line of research as a whole, there are a few suggestions I would like to offer. Regarding the fluid shear stress *in vitro* portion of this project, for all of the eventual 15-25 genes of interest to be verified by qRT-PCR, I believe it would be prudent to also confirm correspondence of mRNA expression fold change to that at the protein level. It is of course well known that mRNA expression will not always correlate with protein expression of the same gene [376], for a variety of reasons, such as post-translational mechanisms involved in turning mRNAs into proteins possessing differences/defects, epigenetic expression modification of the subsequent DNA sequences through methylation or histone deacetylation processes [377], or proteins possessing different half-lives than do their mRNA counterparts [378]. Whatever the case, if any of these consequences is dissimilar between normal and AIS patients, it could be of profound importance to knowledge of the disease. Secondly, real-time confocal imaging of patient bone cells' calcium ion flux in response to applied fluid shear stress would be a logical next step and complement to this work. Frequently, this type of experiment has been a part of such studies in the past on this theme [237, 379, 380], and as we have already noted in the first chapter, calcium ions are key first responders to many forms of mechanical stress; they are particularly so for fluid shear stress. Any alteration in this early response mechanism in AIS patients may be a clue, therefore, as to further changes downstream in the chain of signalling events. Finally, one recommendation to advance the

in vivo research presented in Chapter 3 would be to add in future studies the tracking of certain patient environmental factors; probably this would be most easily and efficiently done through the form of a patient questionnaire of some sort. Again, as we have seen in the introduction, scoliosis has a variety of potential lifestyle and environmental risk factors associated with it. Several of these lifestyle choices mentioned therein (ballet/gymnastics, regular playing of musical instruments, even dietary consumption of estrogen-heavy foods) possess elements that could very well alter the long-term biomechanical profile of a particular subject, perhaps affecting our gotten responses. Thus, it would be well to monitor these confounding effects, if present, for any trends that might be generated in the resulting OPN responses. This is a dimension that can be added fairly easily and advantageously to future studies without too much cost or difficulty, even with the much larger patient cohorts that we envision recruiting.

6.3 Limitations

There were, to be sure, a few limitations on a technical level faced in going through the work done on this project. These were not insurmountable obstacles and should not be for future studies, but certainly represented inconveniences that hampered the ease with which the experimental protocols could be carried out, and should be addressed at the earliest opportunity before new efforts are strenuously undertaken. *In vivo*, when studying mechanotransduction with the aid of the ABR Therapeutic Massager device, it must always be kept in mind that in terms of manpower and equipment, the protocol is somewhat demanding. A nurse or other qualified personnel must always remain on hand to draw blood at the required intervals. Moreover, time and space availability in the hospital centre coinciding with those of our patients was sometimes a hindrance, though admittedly for the scale at which the work in Chapter 3 was carried out, this turned out to be not too onerous. However, if the cohort is to be expanded in future as we envisage, these matters may become severe rate-limiting constraints in terms of project speed. *In vitro*, the main outstanding technical issue concerns the size of parallel plate flow chamber. We recall that the commercially available flow chamber from Glycotech we employed in this study was designed for use with 35 mm diameter cell culture dishes. Unfortunately, this size of dish makes the task of harvesting sufficient amounts of RNA or

any other molecular entity from a particular sample for subsequent experimentation a very slow and delicate one, as there simply are not that many cells to work with in the first place, even at 100% confluence. Any losses or errors incurred in the process of extraction almost invariably forced a repeat of the flow/no flow experiment protocol from the beginning. In addition, to ensure a sufficient stock of RNA for the recommended 15-25 qRT-PCR gene verifications to come, several technical replicates of flow/no-flow experiments would need to be performed for every sample if the 35 mm flow chamber continues to be used; a highly inefficient process. Since there is no commercially available parallel plate flow chamber on the market today designed to work with cell culture plates larger than 35mm in diameter, I recommend that a new one be designed and machined from scratch for use with larger plates (preferably 100 mm in diameter) in the first instance. Likely this is most easily done by dimensionally "scaling up" the present Glycotech design and features. Though this will compel the recalculation of all fluid mechanic parameters in order to ensure correct usage, this is but a small price to pay for the enormously enhanced efficiency due to increased RNA yields per sample.

6.4 Conclusion

In conclusion, this Masters project allowed the characterization of mechanotransduction phenomena in humans affected by AIS, both *in vivo* at the global level as well as *in vitro* at the cellular level. We saw a novel connection between mechanical force externally applied to the body and circulating OPN levels in AIS patients of differing severities. Furthermore, we noted the fascinating potential that this has to one day possibly become the basis of a diagnostic test for scoliosis. We then observed the innovative use and utility of fluid shear stress-based approaches in human mechanotransduction study, which aided in demonstrating that there appear to be a great many fundamental differences in cellular mechanical signal processing mechanisms between unaffected individuals and different functional subgroups of affected AIS individuals. All told, the body of work presented in this project has probable consequence both in AIS pathogenesis theory as well as in clinical approaches to treatment of the disease. We fervently believe that these results will excite and stimulate future

researchers in years to come with new ideas and inspirations for further study along these lines.

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Appendix:

Copies of Ethics Certificates for Projects Presented in this Thesis

Le 18 janvier 2012

Monsieur Alain Moreau
Centre de recherche
Étage A Bloc 7

CHU Sainte-Justine
Le centre hospitalier universitaire mère-enfant

Pour l'amour des enfants

Université de Montréal

OBJET: Titre du projet: Méthodes diagnostiques pour la scoliose idiopathiques de l'adolescent et autres pathologies reliées aux déformations du rachis: Nouvelle technologie pour la détection et le contrôle de la progression / Methods of diagnosis adolescent idiopathic scoliosis and related syndromes causing spinal deformities: Innovative tools for early detection and assessment of risk of progression.

No. de dossier: 2380

Responsables du projet: Alain Moreau Ph. D., Investigateur principal. Collaborateurs: Hubert Labelle, M.D., Benoit Poitras, M.D., Charles-Hilaire Rivard, M.D., Guy Grimard, M.D., Stefan Parent, M.D., Jean Ouellet, M.D., Benoit Morin, M.D., Jean-Marc M. Thiong, M.D., Constantin Stanciu, M.D., Marie-Andrée Cantin, M.D., Anne-Marie Laberge, Ph.D. et Florina Moldovan, M.D./Ph.D., DaShen Wang, M.D./Ph.D., Françoise Maheu, Ph.D., Ginette Lacroix, Inf. Coord. et Ginette Larouche, Inf.

Monsieur,

Votre projet cité en rubrique a été renouvelé par le comité d'éthique de la recherche en date du 16 janvier 2012. Vous trouverez ci-joint la liste des documents approuvés ainsi que vos formulaires d'information et de consentement estampillés dont nous vous prions de vous servir d'une copie pour distribution.

Tous les projets de recherche impliquant des sujets humains doivent être réexaminés annuellement et la durée de l'approbation de votre projet sera effective jusqu'au **16 janvier 2013**. Notez qu'il est de votre responsabilité de soumettre une demande au Comité pour le renouvellement de votre projet avant la date d'expiration mentionnée. Il est également de votre responsabilité d'aviser le Comité de toute modification à votre projet ainsi que de tout effet secondaire survenu dans le cadre de la présente étude.

Nous vous souhaitons bonne chance dans la continuité de votre projet et vous prions de recevoir nos meilleures salutations.

Patrick A. Gogognon, éthicien
Responsable du suivi annuel du Comité d'éthique de la recherche

PG/mhl

3175, Côte-Sainte-Catherine
Montréal (Québec)
H3T 1C5

Le 01 décembre 2009

Dr Alain Moreau
Centre de recherche
Étage A Bloc 7

OBJET: Titre du projet: Déterminant moléculaire et génétique de la scoliose idiopathique: Rôle de l'ostéopontine et sa méchanotransduction dans l'initiation de la scoliose idiopathique et la progression des déformations rachidiennes

No. de dossier: 2961

Responsables du projet: Alain Moreau Ph. D., chercheur responsable au CHU Sainte-Justine.

CHU Sainte-Justine
Le centre hospitalier universitaire mère-enfant

Pour l'amour des enfants

Université de Montréal

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Cher Docteur,

Votre projet cité en rubrique a été approuvé par le comité d'éthique de la recherche en date d'aujourd'hui. Vous trouverez ci-joint la lettre d'approbation du Comité, la liste des documents approuvés ainsi que vos formulaires d'information et de consentement estampillés dont nous vous prions de vous servir d'une copie pour distribution.

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Nous vous souhaitons bonne chance dans la réalisation de votre projet et vous prions de recevoir nos meilleures salutations.

Jean-Marie Therrien, Ph.D., éthicien
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