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Variable Number of Tandem Repeat Polymorphisms (VNTRs) in the ACAN Gene Associated with Pectus Excavatum

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Key Words:	Aggrecan, Cartilage, Connective Tissue, Haller Index, Pectus Excavatum

**Variable Number of Tandem Repeat Polymorphisms (VNTRs) in the *ACAN* Gene
Associated with Pectus Excavatum**

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The following authors have stated there is no conflict of interest that has biased this work.

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The study sponsors had no role in study design, collection, analysis and interpretation of data; writing the report or in the decision to submit the report for publication.

For Review Only

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ABSTRACT

We investigated polymorphisms in the variable number of tandem repeat polymorphisms (VNTR) regions of the *ACAN* gene in 154 patients with pectus excavatum that required surgery. To our knowledge, this is the first study to examine correlation of a functional VNTR genotype of cartilage with pectus excavatum. Patients, non-affected family members, and controls were genotyped by PCR and the number of VNTRs identified. We observed that patients had significantly more 27 repeats, and significantly less 25 repeats, than controls and parents. We examined VNTRs with severity of disorder and found no correlation between *ACAN* genotype, repeat numbers, and severity. Subgroups in the pectus population were observed. There were a number of unique differences in these subgroups, especially in patients with a Marfan clinical phenotype, where there was a correlation of increased number of VNTRs with phenotype. Conversely, in the affected females, there was an inverse correlation between *ACAN* VNTRs and gender. Females also tended to present with a more severe phenotype compared to males. This is the first description of genetic polymorphisms in a candidate gene for pectus excavatum. Overall, there are observed associations between the VNTR regions of *ACAN*, a potential candidate gene, with pectus excavatum.

Key words

Aggrecan, cartilage, connective tissue, Haller index, pectus excavatum,

INTRODUCTION

Pectus excavatum is the most common chest wall deformity, affecting 1 in 400 live births in the United States (1). It occurs commonly in Caucasian males, and is characterized by a depression of the anterior chest wall and sternum that, when severe, result in compromised cardiac and respiratory function (2,3). Pectus excavatum is associated with other anomalies of the musculoskeletal system (4). Abnormal levels of zinc, magnesium and calcium have been reported (5), as have immunological abnormalities, collagen synthesis disturbance in skin fibroblasts, and abnormal structure of type II collagen of costal cartilage (6). Costal cartilages of patients with pectus excavatum are overgrown and the occurrence of sunken chest in other known disorders of connective tissue, such as Marfan syndrome, suggests that defects in the extracellular matrix may be an underlying cause (7). The causes of pectus excavatum remain unclear, although genetic factors appear to play a part (4, 8, 9, 10, 11). Pectus excavatum is a multifactorial trait with some families demonstrating clear autosomal dominant, recessive and sex-linked inheritance patterns. Pectus excavatum falls into the category of complex disorders. The many variants of pectus excavatum may be explained by the interaction of multiple genes, but no one gene has been identified short of *FBNI* in individuals with Marfan syndrome and *COL1A1*, *COL3A1* and *COL5A1/A2* in individuals with Ehlers Danlos syndromes.

The possibility that defects in costal cartilage underlie pectus excavatum led us to investigate the occurrence of polymorphisms in the variable number of tandem repeats (VNTRs), a functional region of the aggrecan (*ACAN*) gene. Aggrecan significantly contributes to the underlying properties of cartilage and could therefore be considered a candidate gene for this disorder (12).

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3 The CS1 domain of the *ACAN* gene exhibits length polymorphisms due to a variable
4 number of repeats, 19 amino acids in length. Each repeat acts as an attachment site for
5 chondroitin sulphate (13). The presence on aggrecan of a large number of highly charged
6 chondroitin sulphate chains generates an osmotic swelling pressure important in maintaining
7 structural integrity of cartilage. We hypothesized that variation in number of repeat sequences
8 outside of the normal reported range of 26-28 (13, 14) would result in a concomitant change in
9 chondroitin sulphate anchorage sites and compromised structural characteristics of patient
10 cartilage.
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22 The objectives of this study were to I) determine the prevalence of *ACAN* VNTRs in a
23 patient population displaying cartilaginous anomalies, II) correlate VNTR polymorphisms to
24 severity of the disorder and III) identify subgroups in our patient population and relate VNTR
25 polymorphisms to subgroup. This is the first description of genetic polymorphisms in a
26 functional region of a candidate gene from patients with pectus excavatum.
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37 MATERIALS AND METHODS

38 *Study Population*

39 A total of 154 patients attending the pectus excavatum clinic of the Children's Hospital of The
40 King's Daughters (CHKD), Norfolk, VA were recruited from across the United States. The
41 severity of chest wall deformity was characterized by computed tomography of the chest to yield
42 the Haller Index (Figure 1) (2, 15, 16). Large Haller indices indicate a more severe depression of
43 the sternum. Patients were considered severe enough to warrant surgical repair for their disorder,
44 and ranged in age from late teens to early twenties at surgery. Five to ten milliliters of blood was
45 drawn from consenting patients, thirty one interested family members, and from 37 unaffected
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3 individuals with no family history, with full IRB approval of Eastern Virginia Medical School.
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5 DNA was extracted from each sample (QiagenQIAmp blood kit) and quantified by
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7 spectrophotometry (Nanodrop ND1000 spectrophotometer). With IRB approval, DNA was also
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9 extracted from chondrocytes isolated from discarded costal cartilage of patients undergoing
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11 surgical repair by the Ravitch procedure at the Hospital for Sick Children, Toronto, Canada (10
12
13 patients). DNA extraction was by the Qiagen DNeasy Tissue kit and quantified as above.
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16 17 *Determination of ACAN VNTR polymorphisms*

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19 Forward TAGAGGGCTCTGCCTCTGGAGTTG and reverse primers
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21 AGGTCCCCTACCGCAGAGGTAGAA were used to amplify DNA of the aggrecan VNTR
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23 region (13, 14). PCR was performed using PCR Supermix High Fidelity (Invitrogen) with
24
25 200nM dNTPs, 100ng genomic DNA, and 5% DMSO. Amplification was carried out for 29
26
27 cycles by denaturation at 94°C for 2min, followed by denaturation at 94°C for 1min, annealing at
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29 66°C for 1min, and extension at 72°C for 2min, with a final extension at 72°C for 10min.
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31 Fragments were separated on 5% TBE polyacrylamide gel and stained with SYBR Green.
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33 Fragments were sized using a 123bp ladder and 3 lymphoblast cell lines of known VNTR sizes;
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35 GM07057, alleles 33/27, GM10851, alleles 25/22, and GM10859, alleles 28/13 were obtained
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37 from the NIGMS Human Genetic Cell Repository, Coriell Institute for Medical Research,
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39 Camden, NJ. (14).
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45 46 *Statistical analysis.*

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48 All analyses were performed using SPSS for Windows (version 17.0). Chi-square analysis was
49
50 computed to evaluate the distribution of VNTR alleles for *ACAN* among patients vs. controls and
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52 patients vs. family members in order to test for potential inheritance differences among these
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54 groups. Independent *t*-tests were also computed to test differences among male vs. female
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3 patients, presence vs. absence of Marfan phenotype, and Caucasian vs. other race category on
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5 Haller Index. Correlations were computed to estimate the relation between the sum of the allele
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7 scores for the *ACAN* VNTR's and the Haller Index.
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10 RESULTS

11 *Study Population.*

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13 A population sample of 154 patients with pectus excavatum consisted of 96% Caucasian of
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15 whom 83% were male. Non-Caucasians included Hispanic (2 males), Asian (1 male, 1 female),
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17 Middle Eastern (1 male), and African American (1 male). The Haller index for 131 patients
18
19 ranged from 2.5 to 11.2, with a median value of 4.4 and an average of 4.88. Five patients were
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21 repeat surgeries with 4/5 having Haller indices below the average of 4.88 (ranging from 3.4-8.5)
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23 suggesting that surgical outcome and high Haller index are not correlated. A sub-group of
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25 patients exhibiting characteristics of Marfan syndrome in whom the diagnosis is not proven were
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27 identified and referred to as patients with the "Marfan phenotype."
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33 *Aggrecan Gene VNTR Polymorphisms.*

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35 Genotyping of the *ACAN* VNTR identified 15 alleles whose frequency is shown in Table 1. The
36
37 observed number of repeats ranged from 19 to 34, with alleles 25-28 accounting for 94%, 78.3%
38
39 and 84.7% respectively in patients, parents and controls. Allele distribution was different among
40
41 patients, parents and control groups ($\chi^2=48.58$, $p<.009$) such that the patient sample had 0.43 and
42
43 0.47 fold fewer 25 repeats ($\chi^2=7.41$, $p<.025$) and 1.5 and 1.7 fold more 27 repeat alleles
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45 ($\chi^2=145.32$, $p<.001$) compared to controls and parents (Table 1). Clearly these data demonstrate
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47 a specific association with VNTR variations among patients, parents, and control populations.
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53 The observed range of allele repeats allows for 135 genotype combinations, of which 26
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55 different genotypes were observed in patients. Coinciding with the high number of allele 27
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3 repeats in patients (41% of all alleles), this allele was present in 66% of genotypes. The
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5 frequency of 27/27 homozygote in patients is 0.112 compared to 0.033 and 0.028 in parents and
6
7 controls respectively.
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10 *Correlation of ACAN VNTR with Haller index.*

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12 To determine a correlation of severity with *ACAN* VNTR allele number, allele score was
13
14 plotted against Haller index. Allele score is defined as the sum of allele repeats found in the two
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16 *ACAN* alleles of each individual (13). There is no apparent distribution bias of allele score with
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18 increased Haller index (Figure 2) suggesting that a specific number of VNTRs does not
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20 predispose to increased severity in pectus excavatum. Similarly, when genotype is compared to
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22 Haller index (Figure 3), there is no apparent bias of genotype with Haller index, suggesting that a
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24 specific *ACAN* VNTR genotype is not associated with severity.
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30 Five patients had undergone previous surgery for repair, suggesting a small subgroup
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32 with increased risk. Table 2 compares allele sizes to Haller index. Although the population is
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34 small, 80% of *ACAN* allele repeats 25 to 28 and 20% of allele repeats 21 and 22 are present in
35
36 this subpopulation compared to 94% and 3% respectively in the overall patient population. Thus,
37
38 there is a tendency for lower number of VNTRs in patients with repeat surgeries.
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41 *Comparisons of ACAN score in males and females to Haller index.*

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43 When separated by sex, the Haller index showed a significant difference (males Mean= 4.706 vs
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45 females Mean=5.691, $p=0.039$), suggesting that females present with a more severe deformation
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47 compared to males. Comparing *ACAN* score to Haller index, males showed a correlation
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49 coefficient of -0.102 compared to -0.270 in females, suggesting a stronger correlation with
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51 decreased number of *ACAN* VNTRs in females than males.
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55 *Comparison of ACAN scores to Haller index in patients with a Marfan phenotype.*

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3 There was no significant difference in Haller index between Marfan phenotype pectus excavatum
4 patients (5.525) compared to non-Marfan phenotype patients (4.797) ($p=0.267$). However,
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6 comparing *ACAN* score to Haller index, there is a stronger positive correlation in Marfan
7
8 phenotype patients (0.321) ($p=0.264$) when compared to non-Marfan phenotype patients (-0.161)
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10 ($p=0.089$), suggesting an increased number of *ACAN* VNTRs in Marfan phenotype patients.
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15 We have examined the inheritance of *ACAN* VNTRs in patients with pectus excavatum to
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17 determine variation in the number of VNTRs associated with this disorder and were able to show
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19 significant differences in the occurrence of alleles with 25 and 27 repeats. We were able to
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21 identify sub-groups in our patient population and show unique differences in these subgroups,
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23 especially in patients with a Marfan clinical phenotype where there was a strong correlation with
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25 increased number of *ACAN* VNTRs compared to non-Marfan phenotype patients. There was,
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27 however, no evidence of a correlation in number of VNTRs with severity of the disorder.
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34 DISCUSSION

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36 Pectus excavatum is the most common inherited chest wall deformity and clinically
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38 important due to associated heart, lung and psychological problems. Specific gene contributions
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40 relative to the severity of the disorder are unknown. Our patient sample was consistent with the
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42 race and gender bias observed for this disorder (2). Haller indices characterized more severe
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44 patients and all required surgery (17), therefore our results reflect those patients only, as the full
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46 range of this disorder (mild to moderate cases not requiring surgery) was not studied. We
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48 investigated the inheritance and size of polymorphisms in VNTR regions of the candidate gene
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50 *ACAN* in this sample population of patients with pectus excavatum from across the United
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3 States. We determined correlation coefficients between *ACAN* and Haller index to identify
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5 associations of number of VNTRs to severity.
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8 Mutations in the *ACAN* gene are associated with skeletal dysplasias (18, 19). Patients
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10 with pectus excavatum commonly exhibit scoliosis, and *ACAN* has been investigated as a
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12 candidate gene in familial idiopathic scoliosis (20, 21). The number of *ACAN* VNTRs determines
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14 the number of GAG side-chains and thus hydration capacity. Smaller repeat sequences may
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16 result in mechanical shearing and tearing (22), and are associated with rheumatoid arthritis and
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18 spinal disc degeneration (19, 23). We observed a distribution of repeat numbers consistent with
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20 previously published work (13, 14, 24). Interestingly, alleles of 27 repeats have been suggested
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22 to have a protective effect against osteoarthritis in homozygous, but not heterozygous subjects
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24 (24). Although our patient sample showed significantly more alleles of 27 repeats, costal
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26 cartilage is not exposed to the same mechanical forces encountered in articular cartilage where
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28 the number of repeats may have a more significant role. The presence of significantly more 27
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30 repeats may be evidence of a preferential transmission of this VNTR polymorphism, suggestive
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32 of possible linkage disequilibrium. In agreement with Roughley *et al* (13), the majority of patient
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34 *ACAN* VNTR allele scores fall within the range of 50-58 for both males (92%) and females
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36 (89%) and was not associated with severity. Similarly, there was no association of patient
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38 genotype with severity suggesting combinations of different allele sizes are not significant.
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46 Patients with pectus excavatum show phenotypic variation and this study has identified
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48 subgroups where a genetic component may be influential (gender, ethnicity, Marfan phenotype,
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50 and patients who have undergone repeat surgeries), however these groups are currently too small
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52 to draw strong correlations. Females (16% of patients), a group showing a significant increase in
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54 phenotypic severity compared to males, showed a tendency towards a decreased number of
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3 *ACAN* VNTRs. The apparent higher Haller indices in female patients is of interest, and is
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5 consistent with other multifactorial traits such as cleft lip and palate where there is a higher
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7 threshold for girls to be affected, with more severe effects in females once the threshold is
8
9 exceeded (25).
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12 Marfan phenotype patients are a sub-group showing characteristics of Marfan syndrome
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14 but without fulfilling all criteria of Marfan syndrome (26). These patients (10.4% of patients)
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16 showed an increase in *ACAN* repeat numbers, which is inconsistent with the reduced number of
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18 VNTRs in female patients.
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21 Repeat surgeries are the smallest subgroup (3.2% of patients). There was no correlation
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23 between Haller index and surgical outcome in these patients; however, there is a tendency of a
24
25 decreased number of *ACAN* VNTR repeats in this group. A smaller number of repeats are
26
27 consistent with the hypothesis of reduced attachment sites for chondroitin sulphate and reduced
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29 osmotic pressure which along with other factors may result in weakened cartilage.
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33 The lack of consistency in correlation within subgroups with specific VNTR
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35 polymorphisms suggests that VNTRs of *ACAN* are not strong correlates with this disorder;
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37 however subgroups are currently small in number.
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41 In conclusion, the VNTRs of *ACAN* demonstrate unique differences in patients, parents
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43 and control subjects. Although the *ACAN* VNTR is biologically functional, variations in other
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45 parts of the gene cannot be ruled out (27). This study has investigated and identified variation in
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47 a functional VNTR, a useful marker for first-pass analysis. Polymorphisms of SNPs will allow a
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49 more refined description in the inheritance of this, and other candidate genes, in the role of
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51 inherited chest wall deformities. Importantly, this is the largest study to date examining specific
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53 molecular contributions of a candidate gene to pectus excavatum.
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Legends

Figure 1. CT scan demonstrating dimensions (L2/L1) to calculate the Haller index. Cardiac compression and displacement are evident.

Figure 2. Correlation of aggrecan allele score and Haller index in male (squares) and female (diamonds) patients.

Figure 3. Correlation of aggrecan genotype (VNTR repeats/allele) with Haller index in male (squares) and female (diamonds) patients.

Table 1. Allele frequency of VNTR repeats in the aggrecan gene in patients, parents and controls. * denotes significant difference in frequency between patients and parents or controls.

Table 2. *ACAN* genotypes from patients who have had 2 or more surgeries compared to Haller Index.

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Tables

Table 1. Frequency of *ACAN* VNTR alleles.

Repeat's	19	20	21	22	23	24	25	26	27*	28	29	30	31	32	33	34
PE	0.003	0.007	0.01	0.02	0.003	0.007	0.078	0.3	0.405	0.157	0.034	0	0	0.003	0.003	0.003
Parents	0	0.017	0	0.067	0	0.033	0.183	0.133	0.267	0.2	0.05	0	0	0.017	0.017	0.017
Controls	0	0.014	0.042	0	0	0.028	0.167	0.361	0.236	0.083	0.042	0.014	0	0	0.014	0

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Table 2. Genotypes of patients who have undergone repeat surgery.

<i>ACAN</i>	Haller Index
22/28	3.4
27/28	4.3
25/27	8.5
21/28	3.9
26/29	4.6

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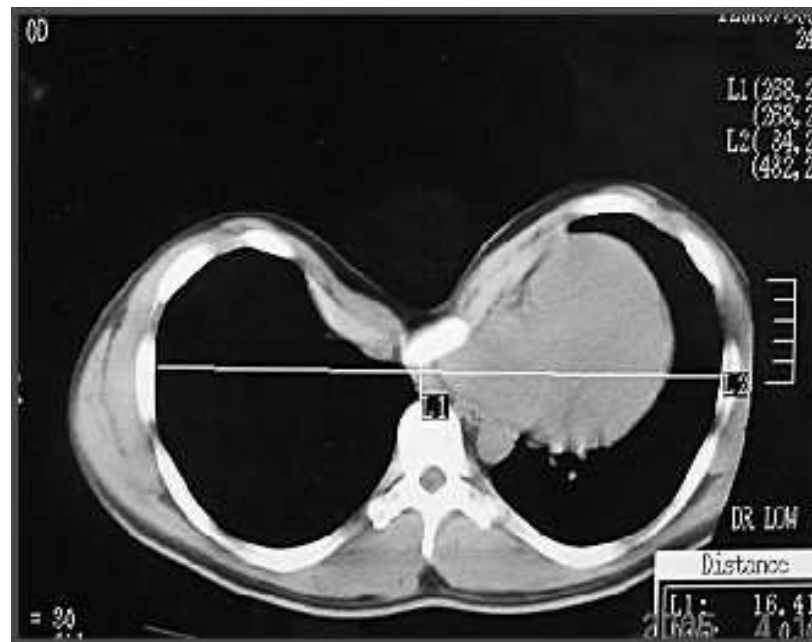


Figure 1. CT scan demonstrating dimensions (L2/L1) to calculate the Haller index, cardiac compression, and displacement.

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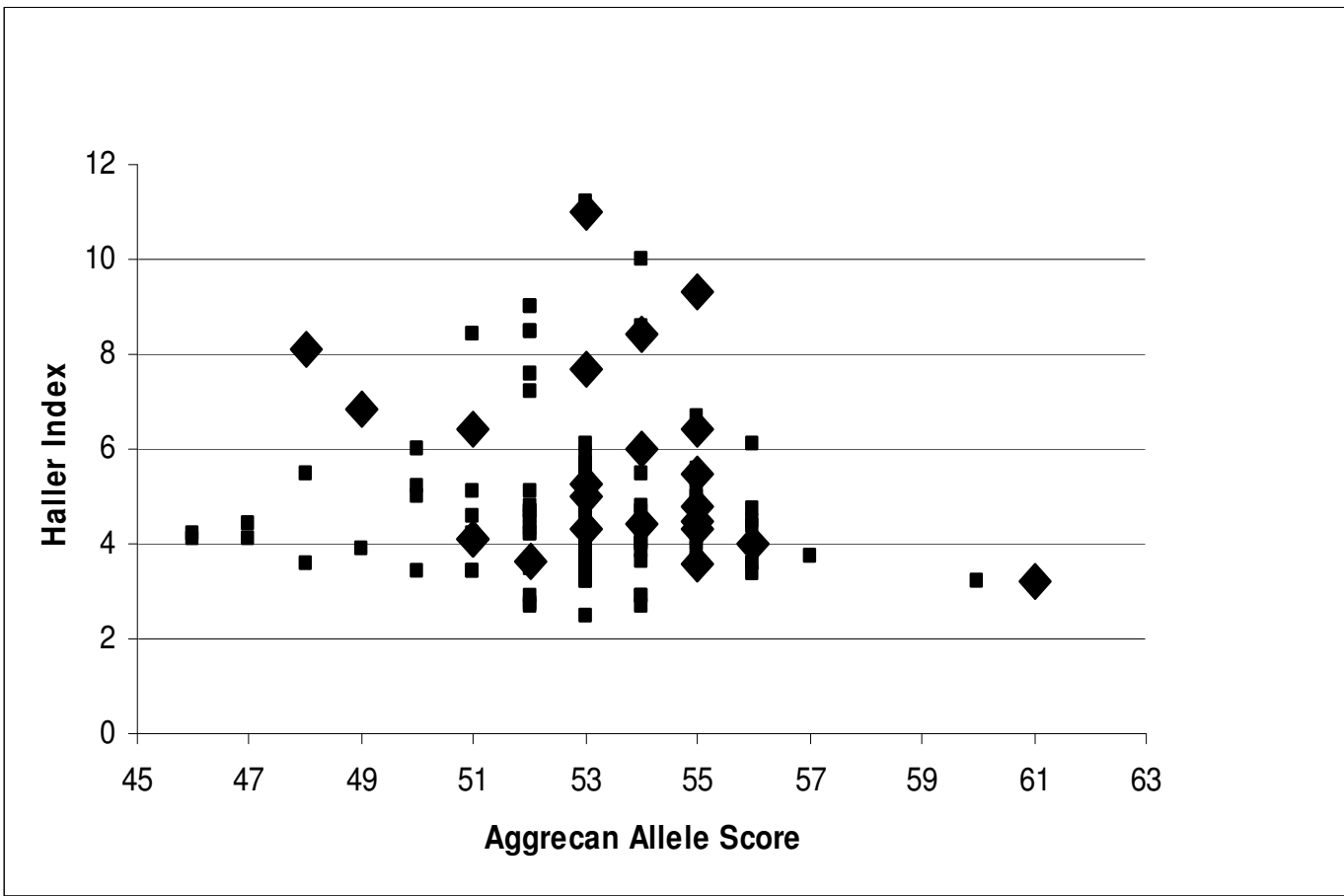


Figure 2. Correlation of aggreacan allele score and Haller index in male (squares) and female (diamonds) patients.

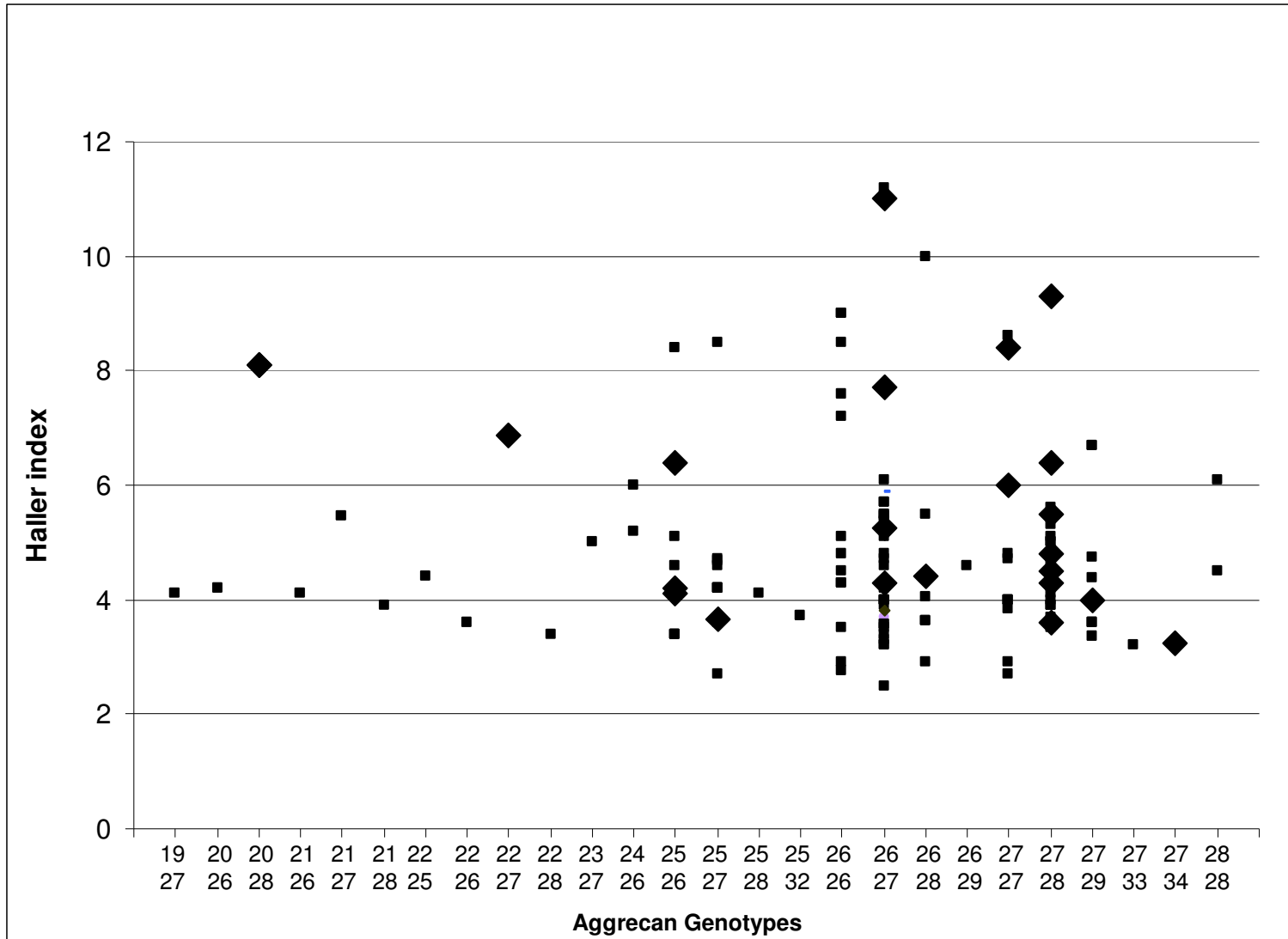


Figure 3. Correlation of aggrecan genotype (VNTR repeats/allele) with Haller index in male (squares) and female (diamonds) patients.