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Part 2

Development of an atrioventricular septal defect
Chapter 2.1

How normal is a “normal” heart in fetuses and infants with Down syndrome?


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ABSTRACT

Background
Congenital heart disease is present in 44–56% of fetuses with Down syndrome (DS). There are, however, signs that hearts in DS without apparent structural heart defects also differ from those in the normal population. We aimed to compare the atrioventricular (AV) septum and valves in 3 groups: DS without AV septal defect (DS noAVSD), DS with AVSD (DS AVSD) and control hearts.

Methods
The ventricular septum, membranous septum and AV valves were examined and measured in histological sections of 15 DS no-AVSD, 8 DS AVSD and 34 control hearts. In addition, the ventricular septum length was measured on ultrasound images of fetal (6 DS AVSD, 9 controls) and infant (10 DS no-AVSD, 10 DS AVSD, 10 controls) hearts.

Results
The membranous septum was 3 times larger in DS no-AVSD fetuses compared to control fetuses, and valve dysplasia was frequently (64%) observed. The ventricular septum was shorter in patients with DS both with and without AVSD, as compared to the control group.

Conclusion
DS no-AVSD hearts are not normal as they have a larger membranous septum, shorter ventricular septum and dysplasia of the AV valves as compared to control hearts.
INTRODUCTION

Congenital heart disease (CHD) occurs in 44–56% of fetuses with Down syndrome (DS), most commonly in the form of atroventricular septal defects (AVSDs) [1, 2]. In AVSDs, the membranous septum is partly or completely absent. During normal development, several structures contribute to the formation of the membranous septum, which consists of an atroventricular (AV) and an interventricular part. These structures include the endocardial cushions present in the AV canal (also contributing to the putative AV valves), the so-called dorsal mesenchymal protrusion (DMP) [3] (also known as vestibular spine, a mesenchymal protrusion at the base of the atrial septum), and an extension of cushion tissue covering the lower rim of the interatrial septum, known as the mesenchymal cap. The AV part of the membranous septum is positioned between the left ventricle and right atrium, and the interventricular part is positioned between the left and right ventricles [4]. AVSDs cover a spectrum of heart anomalies with a common AV junction. In the case of a complete AVSD, a common AV valve, with 2 bridging leaflets, is present between the atria and ventricles with shunting occurring at both the atrial and ventricular level. When the bridging leaflets are attached to the atrial or ventricular septum (partial AVSD), shunting can solely take place at the ventricular or atrial level, respectively.

In addition to AVSD, patients with DS may present with other septal anomalies, such as aneurysms of the membranous ventricular septum [5] and inlet ventricular septal defects [6]. Although half of the patients with DS do not present with CHD, there are signs that the hearts of these fetuses, in particular the AV valves, are not normal. Tricuspid regurgitation during prenatal echocardiography is associated with DS [7, 8], and adults with DS without overt CHD show a high frequency of mitral valve prolapse [9, 10].

The above data suggest that fetuses with DS without overt congenital heart defects are still at risk of developing cardiac abnormalities. Furthermore, the etiology of maldevelopment of the (AV) membranous septum in patients with DS seems largely unexplained to date. The question arises whether hearts designated as normal in DS may in fact be abnormal, and if so, what causes this abnormality? Interestingly, recent studies on cerebellar and neural cells in DS have shown a response deficit to the mitogenic effects of sonic hedgehog (SHH), a gene involved in establishing cell fates at multiple points during development and known to contribute to cardiac septation [11, 12]. SHH is therefore one of the candidate genes for heart abnormalities in DS patients.

The aim of the current study was to evaluate the hearts of fetuses with DS without overt CHD, focusing on: (1) morphology and size of the membranous septum (AV and interventricular part), (2) morphology and the size of the ventricular septum and (3) morphology of the AV valves.
METHODS

Light microscopy of serial sections

This study was performed in accordance with the local ethics committee and the Dutch and Polish regulations for proper use of human tissue for medical research purposes. Serial sections of postmortem fetal hearts were selected from the Leiden Collection (Department of Anatomy and Embryology, LUMC, the Netherlands) and the heart collection of The Medical University of Warsaw in order to examine the fibrous tissue, the interventricular septum and the valvular structures. The age of the specimens ranged from 10 + 0 weeks’ gestational age (GA) to 3 days postpartum.

For the current study, 3 groups of hearts with known karyotype were selected. Group 1 included 15 human hearts with DS without septal defect (GA 12–22 weeks, further referred to as DS noAVSD); group 2 contained 8 human hearts with DS and AVSD (GA age 11–18 weeks, further referred to as DS AVSD); group 3 contained 34 structural normal human hearts with normal karyotype (GA 10 weeks to 3 days postnatally, further referred to as control hearts).

All fetuses were obtained either following pregnancy termination or miscarriage after fetal or neonatal death. The hearts were sectioned in transverse, frontal, or sagittal planes. The histological and immunohistochemical tissue markers used in these serial sections were HE (to analyze general anatomy), resorcin-fuchsin-iron (staining fibrous tissue), hematoxylin-picric acid-thiazin red (modified Van Gieson’s stain, staining fibrous tissue and myocardium) and troponin I (staining myocardial tissue, 1/250 SC-15368, Santa Cruz Biotechnology Inc., Dallas, Tex., USA). To assess a possible role of the SHH pathway in septation and valve development, selected hearts were also stained with antibodies for SHH (1/100 SC-1194, Santa Cruz Biotechnology) and its downstream effector Gli1 (1:50 AB-49314; Abcam, Cambridge, Mass., USA). Primary antibodies were dissolved in PBS-Tween-20 with 1% bovine serum albumin (Sigma Aldrich, St. Louis, Mo., USA). Between subsequent incubation steps, all slides were rinsed in PBS (2×) and PBSTween-20 (1×). The slides were incubated with the secondary antibodies for 60 min: for troponin I and Gli1 with 1/200 goat antirabbit biotin (BA-1000, Vector Laboratories, Burlingame, Calif., USA) and 1/66 goat serum (S1000, Vector Laboratories) in PBSTween-20, and for SHH with 1/200 horse anti-goat biotin (BA-9500 Vector Laboratories) and 1/66 horse serum (S-2000, Vector Laboratories) in PBS-Tween-20. To amplify the signal, all slides were incubated with ABC reagent (PK6100, Vector Laboratories) for 40 min. For visualization, the slides were incubated for 10 min with 400 μg/ml 3,3’-diaminobenzidine tetrahydrochloride (D5637, SigmaAldrich) dissolved in Tris-maleate buffer pH 7.6 to which 20 μl of H 2 O 2 was added. Counterstaining was performed with 0.1% hematoxylin (Merck, Darmstadt, Germany) for 3 s, followed by rinsing with tap water for 10 min. Finally, all slides were dehydrated and mounted with Entellan (Merck). As staining quality depended on the fixation method of the hearts, we used the smooth muscle cells in the esophagus as positive control for SHH and Gli1 in each heart.
Membranous septum measurements in sections of fetal hearts
The membranous septum was defined as a nonmuscular structure comprising an interven-tricular part between the left and right ventricle and an AV part between the left ventricle and right atrium, as previously described by Allwork and Anderson [4] (figure 1 a, b). In hearts with sufficient four-chamber views (defined as visibility of 2 atria, 2 ventricles and the AV valves) in transverse sections of similar GA (DS no-AVSD, n = 6; DS AVSD, n = 6; control, n = 10), the presence of the AV part of the membranous septum and the ventricular part of the membranous septum was analyzed. The volume of the membranous septum was estimated based on the Cavelieri’s principle [13] . Regularly spaced (49 mm 2 ) points were randomly placed on resorcin-fuchsin-iron- or troponin-stained hearts. The distance between the subsequent sections was between 0.056 and 0.2 mm. Volume measurements were performed using an Olympus microscope with a x40 magnification objective for hearts of embryos 13 weeks.

Echocardiographic assessment of the ventricular septum in fetal hearts
The ventricular septum length was assessed in DS AVSD and control fetuses to compare the findings in the fetal specimens with living fetuses. We examined 2-D ultrasound images, obtained from STIC (spatiotemporal image correlation) volumes, of all cases with DS from a prospectively recorded database of fetuses, which were referred to our center for fetal echocardiography. STIC is a modality to record the heart in 3 directions during one complete heart cycle, which allows the heart to be visualized in any desired plane [14, 15] . This database also contains a series of normal controls. Fetal ultrasound images of 6 DS AVSD fetuses (GA 20 + 0 to 30 + 0 weeks) and 9 randomly selected controls of comparable GA (GA 19 + 4 to 32 + 2 weeks) were available. The ventricular septum was measured on the available four-chamber view from the septal attachment of the tricuspid valve to the endocardium of the apex.

Transthoracic echocardiographic assessment of the ventricular septum in hearts of infants
To gain more insight into the postnatal situation, measurements were also obtained from hearts of live-born infants. Ten DS no-AVSD infants (1 day to 15 years), 9 DS AVSD infants (39 days to 2 years) and 10 control infants (1 day to 15 years) were measured. We selected all cases with DS from a prospectively recorded database of ultrasound images of infants who were referred to our center for echocardiography. No ultrasound recordings were available of older patients with AVSD, as most of these patients undergo surgery at an early age. The ventricular septum was measured from the septal attachment of the tricuspid valve to the endocardium of the apex on the available four-chamber view in the case of sufficient quality (figure 2).

Morphology of the AV valves in sections of fetal hearts
The insertion of the AV valves was studied in specimens in the 3 groups (DS no-AVSD, n = 15; DS AVSD, n = 8; control, n = 34). Furthermore, each heart was assessed for dysplastic features of the
AV valves (plump, irregular contours). The tricuspid valve of a normal heart has a more apical insertion as compared to the mitral valve, also known as ‘differential insertion of the atrioventricular valves’ [16]. A linear insertion (attachment of the AV valves to the septum forming a straight line) is a feature of AVSD when imaging is performed at a specific level [16]. Linear insertion has also been suggested as a landmark for DS without AVSD, although data are limited [17].

**Statistical analysis**

Statistical analysis of the differences in length and volume measurements of the membranous septum between (1) DS no-AVSD hearts, (2) DS AVSD hearts and (3) control hearts was performed with SPSS software (SPSS Inc., Chicago, Ill., USA). A linear regression analysis was performed, and data were corrected for GA. Outcome data as well as GA were log transformed to reach normality, and the antilog was used for the result.

**RESULTS**

**Membranous septum: morphology**

Between 8 and 16 weeks’ GA, neither DS no-AVSD hearts nor the hearts of controls showed an interventricular component of the membranous septum. At 16–28 weeks’ GA, 66% of the fetuses in the control group showed both an interventricular and an AV component of the membranous septum. The remaining 43% only had an AV septum (figure 1 a–c).

In the DS no-AVSD hearts (figure 1 d), more variations in the membranous septum were observed: only 20% presented with both an interventricular and an AV septum, 20% with only an interventricular septum, 40% with only an AV septum and 20% with merely a fibrous continuity between the left and right AV valves. In the DS fetuses with a (complete) AVSD, fibrous tissue was observed at the top of the ventricular septum in 3 cases. In one of these cases, the central part of this fibrous tissue was attached to the ventricular septum as well as to the atrial septum in the four-chamber view immediately beneath the aortic outflow tract (figure 1 e). The shunt was only visible at another level (figure 1 f, g).

In summary, in DS no-AVSD fewer fetuses had both components of the membranous septum as compared to controls, and in DS AVSD a part of the AV septum is present.

**Membranous septum: volume**

Linear regression analysis showed a 2.6 times (p = 0.0001, 95% CI 1.70–3.98) larger volume of the membranous septum in DS no-AVSD as compared to controls. The mean membranous septum volume estimation was 0.19 cm³ in DS no-AVSD hearts (GA 16 ± 4 weeks) and 0.08 cm³ in control hearts (GA 14 ± 6 weeks).
**Figure 1.** The atrioventricular and ventricular components of the membranous septum.

A: Transverse section immediately beneath the aortic outflow tract of a first-trimester control heart with solely an AV (white arrow) component of the membranous septum. B: Transverse section immediately beneath the aortic outflow tract of a second-trimester control heart with an AV (white arrow) and an interventricular (black arrow) membranous septum. C, D: Transverse section immediately beneath the aortic outflow tract in 2 hearts of fetuses with DS no-AVSD showing a longer and plump membranous septum with white arrows indicating the AV component of the membranous septum and the black arrow the interventricular component. E: Transverse section immediately beneath the aortic outflow tract of a heart from a patient with DS AVSD showing a membranous septum [with an AV (white arrow) and an interventricular (black arrow) part] where the central fibrous body attaches to the ventricular septum as well as the atrial septum. F: Same heart as in e in the fourchamber view, more towards the diaphragm. At this level, the complete AVSD can be observed. G: 3-D reconstruction of the same DS AVSD heart with part of the membranous septum (asterisk). Red indicates the great arteries, yellow fibrous tissue and grey ventricular myocardium. Colors refer to the online version only. The section in B is stained with resorcin-fuchsin-iron, all other sections are stained with troponin I. RA = Right atrium; RV = right ventricle; LA = left atrium; LV = left ventricle; LAVV = left AV valve; RAVV = right AV valve.

### Ventricular septum length

Using ultrasound imaging, the ventricular septum length was measured in fetuses and in infants. In fetuses, the ventricular septum length in DS AVSD was 0.7 times the length of controls \((p = 0.001, 95\% \text{ CI } 0.6–0.9)\). Infants with DS with and without AVSD both showed a smaller ventricular septum length compared to controls (DS noAVSD 0.78 times the size of the control, 95% CI 0.65–0.93, \(p < 0.0001\); DS AVSD 0.67 times the size of the control, 95% CI 0.56–0.79, \(p = 0.006\);...
There was no statistically significant difference in septum length between infants with DS with and without AVSD ($p = 0.07$).

**Morphology and insertion of the AV valves**

Control hearts ($n = 34$) showed a differential insertion of the AV valves just below the aortic outflow tract. Fetal DS AVSD hearts ($n = 15$) showed a linear insertion in the four-chamber view just below the aortic outflow tract, as described previously [16]. The AV valves of hearts with DS without septal defect showed either a differential ($n = 12$) or a linear insertion ($n = 3$, with GA 12.3, 14.4 and 22 weeks) in the four-chamber view sections just below the aortic outflow tract. Furthermore, the AV valves of these hearts showed dysplastic features in 4 of the 5 hearts between 8 and 16 weeks’ GA and in 3 of 6 hearts between 16 and 28 weeks’ GA (figure 3). The tricuspid valve appeared more affected than the mitral valve. Dysplastic features of valves were not observed in control fetuses.

**Expression of SHH/Gli1**

In first-trimester hearts, clear SHH expression was detected in the tendon of Todaro (the remnant of the DMP [18]) in control hearts (figure 4 c). This was also observed in DS no-AVSD and DS AVSD hearts (figure 4 d). In first-trimester hearts, Gli1 expression was not observed in the AV valves and septum of control hearts ($n = 5$). In contrast, in DS no-AVSD fetuses, clear expression of Gli1 was present in the dysplastic plump AV valves and membranous septum in 2 out of 4 cases (figure 4 f). In DS AVSD fetuses, no Gli1 expression was observed. In the second-trimester hearts, no clear SHH and Gli1 staining could be detected in any group.

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**Figure 2.** Method of ventricular septal length measurement (white arrow) with transthoracic echocardiography.

Measurements were performed in four-chamber views, from the endocardial border to the septal attachment of the tricuspid valve. A: Control postnatal heart of a 29-day-old infant. B: Heart of a 24-day-old infant with DS no-AVSD. C: Heart of a 51-day-old infant with DS AVSD. RV = Right ventricle; LV = left ventricle.
Figure 3. Plump atrioventricular valves in hearts of patients with DS no-AVSD.

A: Transverse section of a control heart, 12 + 1 weeks, with resorcin-fuchsia-iron (RF) staining showing slim, well-developed tricuspid valve leaflets (asterisks). B, C: Transverse sections of DS no-AVSD hearts (B 12 + 3 weeks, stained with RF; C 20 + 0 weeks stained against troponin I) with plump (asterisks) AV valve leaflets compared to the control heart. RA = Right atrium; RV = right ventricle.

Figure 4. Expression of SHH and Gli1.

A: Transverse section of a control heart with resorcin-fuchsia-iron (RF) staining, showing the tendon of Todaro (rectangle). B: Transverse section of a DS AVSD heart with troponin I staining, showing the tendon of Todaro (rectangle). C: A consecutive slide of the section in A, with SHH expression in the tendon of Todaro. D: A consecutive slide of the section in B with SHH expression in the tendon of Todaro. E: Transverse section of a control heart with Gli1 expression in the lungs (arrow), but not in membranous septum (asterisk) and AV valves. F: Transverse section of a DS no-AVSD heart with increased Gli1 expression in the membranous septum and AV valves (black arrows). RA = Right atrium; RV = right ventricle; LA = left atrium; LV = left ventricle; CS = coronary sinus; SVC = superior vena cava.
DISCUSSION

This study presents an overview of morphological findings in hearts of fetuses with DS without apparent cardiac malformations. This group showed: (1) a larger membranous septum, (2) a shorter ventricular septum, (3) dysplastic AV valves and (4) increased levels of Gli in the AV valves and membranous septum.

DS is associated with CHD, most frequently AVSDs, atrial septal defects, ventricular septal defects and tetralogy of Fallot [19]. AVSD has been described as a spectrum of disease [20] with varying levels of atrial and ventricular shunting. An inheritable shorter AV membranous septum, observed in families of patients with nonsyndromal AVSD, suggests a shorter membranous septum to be an intermediate phenotype towards AVSD [21]. In contrast, in other studies, patients with DS without a septal defect were suggested to have a long membranous septum, indicating a long membranous septum being a sliding scale towards AVSD [22], which is in line with the findings in the current study. This discrepancy might partially be explained by the level in the heart where the largest membranous septum is observed in patients with a normal heart and in AVSD. It has been previously shown that in normal fetal hearts, in a plane equal to the echocardiographic four-chamber view and moving from the outflow tracts in the caudal direction towards the diaphragm, the distance between the insertion of the AV valves to the septum becomes smaller. The distance (i.e. the amount of differential insertion) is maximal in the four-chamber view just below the outflow tracts and smallest in the four-chamber view just above the diaphragm [16].

The larger membranous septum, based on microscopy, and shorter ventricular septum, based on echocardiography, in DS no-AVSD hearts in the current study, may reflect the end of the spectrum of a ‘normal’ heart in DS, which may form a sliding scale with an AVSD, as was postulated by Rosenquist and Sweeney [23]. A smaller inlet/outlet ratio is a feature of hearts with an AVSD, and a higher atrial to ventricular length ratio measured in a four-chamber view has been considered as a detection method for an AVSD [24]. The shorter ventricular septum in DS hearts with and without AVSD measured in our study supports this association. A shorter ventricular inlet septum, as was described previously [25], may hinder fusion of the endocardial cushions (which were shown to have a normal size in patients with an AVSD [26]) with the septum. Furthermore, we encountered in our study a fetal heart with an AVSD, in which a significant part of the membranous septum could still be distinguished. The central part of this fibrous tissue attached to the ventricular septum as well as to the atrial septum in the four-chamber view immediately beneath the aortic outflow tract (figure 1e).Scrolling through the heart more caudally, this central part separated from the top of the ventricular septum and a common valve was visualized. We suggest that this centrally attached part could be regarded as a part of the membranous septum. However, in early pregnancy it is difficult to distinguish the membranous septum histologically from the annulus fibrosis and the valves because together they comprise the fibrous tissue of the heart [27, 28].
Several studies report valvular heart disease in patients with DS, including mitral valve prolapse in the majority of asymptomatic adults [11, 29, 30]. Tricuspid valve regurgitation has been suggested as a useful feature for detection of fetuses with DS [7, 8]. The increased prevalence of tricuspid valve regurgitation is considered to be related to the structural changes associated with DS: decreased number of myocytes, abnormal orientation of myocytes and myofibrils and abnormal connective tissue [31]. The observed dysplastic, plump AV valves in fetal hearts with DS confirm an abnormal development of the AV valves in patients with DS.

The high incidence of AVSD in DS suggests that genes on chromosome 21 are likely to be involved in the development of AVSD. However, not all patients with DS develop an AVSD, suggesting a multifactorial influence [32]. The observed SHH expression in the tendon of Todaro, a structure derived from the DMP and necessary for normal AV septation [33], suggests a similar role of SHH in septation in humans as has previously been described in mice [12, 34]. We observed an increased expression of Gli1, a marker for SHH activity, in AV valves and the membranous septum in fetal hearts in DS without AVSD. Although numbers in the current study are too small to draw valid conclusions, it is tempting to speculate that the increased expression is a result of a previously suggested response deficit of SHH signaling in DS [35]. Increased Gli1 expression in fetuses with DS might also be caused by Yak1-related kinase (Dyrk), which induces Gli1 and is found on the DS critical region and is increased in patients with DS [36, 37]. The extensive Gli1 expression in the mesenchymal AV septum possibly influences cell fate, resulting in reduced myocardialization. Such a mechanism would implicate that inhibitors of Dyrk, currently experimentally used to improve cognitive function of DS patients, might be a focus for research concerning therapy of CHD in DS patients as well [38]. Further studies are required to investigate the role of SHH in heart development in fetuses with DS.

The current study is based on a unique collection of fetal specimens. This however causes limitations, including the small number and variable age of the observed hearts, inherent to the limited availability of human fetal material with known phenotype. Some stainings could not be performed in all hearts due to differences in the quality of the material.

In conclusion, hearts of patients with DS, even without overt CHD, appear to be abnormal in the majority of specimens examined. Hearts of fetuses with DS without septal defects have a different, elongated, membranous septum in comparison to hearts of fetuses with a normal karyotype and show abnormal dysplastic AV valves, which may explain the valve dysfunction observed both pre- and postnatally in patients with DS. Correlation with clinical data is necessary to explore if cardiac follow-up in patients with DS without overt CHD is warranted.
REFERENCES


