Chapter 6
Discussion
We evaluated 43 patients from 38 families with apparently non-syndromic MVSD by Sanger sequencing of six known genes (DLL3, MESP2, LFNG, HES7, TBX6 and RIPPLY2) and by exome sequencing of 15 selected families. We identified a monogenic etiology in 22 patients from 17 families with a diagnostic yield of 45%. Ten patients from 7 families had pathogenic variants in FLNB with SCT, eight patients from 7 families had pathogenic variants in DLL3 with SCD I and one patient had pathogenic variant in MESP2 with SCD II. We also studied two patients with possible pathogenic variant in FLNB with non-syndromic MVSD and one patient with pathogenic variant in PTCH1 with Gorlin syndrome. The largest study of vertebral segmentation defects has reported 73 patients with a diagnostic yield of 16% [Lefebvre et al., 2018]. The most frequent cause of MVSD in this study was due to pathogenic variants in DLL3 and LFNG. However, in the present study pathogenic variants in DLL3 was the frequent underlying cause of MVSD.

6.1. Spondylocostal dysostosis I

Here we characterized eight patients with pathogenic variants in DLL3 and describe phenotypic features which are consistent with the earlier reports, notably pebble beach sign as described by Turnpenny et al., 2003 [Turnpenny et al., 2003; Bonafé et al., 2003; Bulman et al., 2000; Lefebvre et al., 2018; Sparrow et al., 2002; Whittock et al., 2004a]. Our experience suggests that it might be possible to radiologically suspect a DLL3 pathogenic variant in patients with typical multiple vertebral segmentation defects. We further add six novel pathogenic variants to the spectra of variants in DLL3.

Majority of the reported pathogenic variants has been found to occur in epidermal growth factor (EGF) followed by repeat delta-serrate-lag2 (DSL) domain of DLL3 protein. The pathogenic variants in our study were also found to cluster in EGF repeat.
Truncating variants were more commonly seen than missense variants which is in concordant with our study. The variant c.661C>T, was found to be recurrent in our cohort.

Pudgy homozygous mouse has demonstrated that DLL3 is crucial for the normal somitogenesis [Kusumi et al., 1998]. Morphological somite borders and rostral-caudal compartment boundaries were found to be severely affected. Heterozygous pu/+ mice were phenotypically normal. Histology of the vertebral bone itself appeared normal despite gross morphology of the vertebrae being disrupted.

6.2. Spondylocostal dysostosis II

To date, 13 Puerto Rican patients from 12 families and two patients from Lebanese Arab family have been reported with pathogenic variants in MESP2 [Cornier et al., 2008; Whittock et al., 2004b]. The clinical and radiological features of Lebanese Arab patients include short trunk and neck, with severely affected cervical and thoracic vertebrae which were similar to those seen in patients with pathogenic variant in DLL3 [Whittock et al., 2004b]. The radiological features reported in Puerto Rican family had multiple vertebral segmentation defects with prominent pedicles of the vertebrae referred to as tramline sign. There was also posterior fusion of the ribs at the costovertebral junction giving rise to “crab-like” appearance of the thorax. The patient reported in this study with pathogenic variant in MESP2 is concordant with the phenotype seen in affected patients from Puerto Rican families. Therefore, we further reinforce the presence of unique segmentation defects of vertebra and rib anomalies in patients with SCD II and report the first non-Puerto Rican and non-Arab patient with this condition.
All the reported pathogenic variants have been found to be distributed across the MESP2 protein. It is of note that majority of the reported pathogenic variants in MESP2 including the novel variant being reported here are truncating.

Homozygous mutant mice deficient of MESP2 demonstrated severe segmentation defect of the somitic mesoderm [Saga, 2007; Morimoto et al., 2007; Takahashi et al., 2007]. Additionally, the establishment of the polarity of the segment was impaired in the mutant mice. The pudgy mouse and Mesp2 null mouse studies collectively highlight that it is the somite segmentation and patterning that is severely disrupted, without the involvement of any other skeletal or non-skeletal manifestation. Thus, elucidates the pathomechanism of isolated segmentation of vertebrae is SCD.

6.3. Unclassified non-syndromic MVSD

Despite thorough evaluation by systematic Sanger sequencing of six genes in 21 patients and exome sequencing of six patients among them, we did not identify a potential monogenic cause for MVSD. They do not demonstrate any typical radiographic pattern as well. These observations are similar to a recent study by Lefebvre et al., 2018 where 12/73 patients received a molecular diagnosis [Lefebvre et al., 2018]. We concur with their observations on the phenotypes and genotypes of disorders of segmentation of vertebrae. Some of the environmental factors that lead to vertebral segmentation defects are maternal exposure during pregnancy to teratogens like valproic acid, vitamin A and maternal gestational diabetes. Therefore, it is challenging to distinguish majority of the vertebral segmentation defects which do not follow any unique pattern and include a range of non-specific vertebral anomalies. Our findings highlight the clinical and etiological diversity of MVSD, which might even
include multifactorial and non-genetic conditions. Furthermore, several earlier reports also indicate the plausibility of unidentified genetic and non-genetic causes of MVSD [Lefebvre et al., 2018; Sparrow et al., 2006a; Whittock et al., 2004b].

We identified a family with the autosomal recessive inheritance of non-syndromic multiple vertebral segmentation defects likely due to a pathogenic variant in FLNB. Exome sequencing identified a rare missense variant, c.1892C>T in FLNB that segregated in the family. To date, only truncating variants in FLNB are known to cause spondylocarpotarsal synostosis syndrome which has fusion of carpal and tarsal bones in addition to block vertebrae [Krakow et al., 2004; Farrington-Rock et al., 2006; Mitter et al., 2008; Yang et al., 2017; Salian et al., 2018]. The radiograph of spines in affected individuals demonstrate block vertebrae, that typically characterizes spondylocarpotarsal synostosis syndrome as proved by us recently [Salian et al., 2018]. Though we could not perform necessary functional studies, our findings (block vertebrae, absent carpal fusion and biallelic missense variant in FLNB) strongly suggest a causal association between FLNB and MVSD.

6.4. Spondylocarpotarsal synostosis syndrome

Here, we report the first substantial cohort of patients with SCT, adding 10 more patients to ten mutation proven cases from nine families reported earlier. To date, 35 cases with SCT have been reported comprising of 14 males and 21 females. The clinical and radiological features are excellently summarized by Yang et al. 2017 in 35 cases [Yang et al., 2017]. Clinical features include short stature (91%), short trunk (83%), short neck (51%), joint mobility limitation (49%), clinodactyly of the 5th digit (34%), club foot (6%), flat foot (43%), hearing loss (46%), cleft palate (11%), kidney anomalies (6%),
inguinal hernia (11%), heart anomaly (17%), dental enamel anomaly (29%), dysmorphic face (49%), retinal anomalies (9%). Radiological features like thoracolumbar fusions (94%), cervical fusion (54%), scoliosis (86%), lordosis (63%), sacral anomaly (23%), carpal coalition (93%), tarsal coalition (37%), brachydactyly (14%), and bowed humerus (6%) were noted [Yang et al., 2017]. However, it must be noted that majority of the patients (25/35) lacked a molecular confirmation of the diagnosis. Our results further validate short stature, short trunk, vertebral anomalies and carpal and tarsal fusions are defining features of SCT [Langer et al., 1994].

Vertebral segmentation defects are genetically and phenotypically diverse and it may be genetic (syndromic or non-syndromic), multifactorial or environmental. Some of the genetic vertebral segmentation defects include spondylocostal dysostosis types I-VI (caused by pathogenic variants in \textit{DLL3}, \textit{MESP2}, \textit{HES7}, \textit{LFNG}, \textit{TBX6} and \textit{RIPPLY2} respectively), multiple synostosis type I-III (caused by pathogenic variants in \textit{FGF9}, \textit{GDF5} and \textit{NOG}) and Klippel-Feil syndrome (caused by pathogenic variants in \textit{GDF6}). Comparison of radiographs of all the patients with genetic vertebral segmentation defects hitherto has led to the identification of few unique patterns pertaining to different disorders with vertebral segmentation defects. Spondylocostal dysostosis type I has a recognizable pattern of vertebral segmentation defects and has been referred to as “pebble beach sign” [Turnpenny et al., 2007, 2003]. Spondylocostal dysostosis type II or Jarcho-Levin syndrome has a characteristic crab-like appearance of the thorax with tramline sign of the vertebral pedicles [Whittock et al., 2004b; Cornier et al., 2008]. However, vertebral segmentation defects observed in other types of spondylocostal dysostosis does not have a characteristic radiological pattern of vertebral defects.
Reviewing the radiographic features of patients with SCT (from earlier reports and this cohort), vertebral fusion appears to have a unique appearance with unsegmented thoracic vertebrae. Furthermore, the absence of rib anomalies and symphalangism and the presence of carpal and tarsal fusion in SCT distinguishes it from other aforementioned phenotypically overlapping conditions. These features help us to clinically narrow down the diagnosis for further molecular analysis.

The underlying pathomechanism of vertebral anomalies seen in SCT and SCD differs substantially. Knockout mice for SCT and SCD have revealed that the vertebral anomalies arise due to the dysregulation in the maintenance of intervertebral space and failure in the segmentation respectively [Farrington-Rock et al., 2006; Zieba et al., 2016; Lu et al., 2007; Bulman et al., 2000].

Expression study of filamin B in mouse embryo by immunostaining revealed a high expression of protein in chondrocytes of vertebrae and a diminished expression in the intervertebral space [Krakow et al., 2004]. Furthermore, expression was also detected in different transition stages of chondrocytes in the growth plate. This collectively suggests the crucial role of filamin B role in skeletal morphogenesis.

Mouse homozygous for truncating variant in FLNB displayed cervical vertebral fusion and carpal bone fusion [Zheng et al., 2007]. This study has demonstrated that there was mineralization of the extracellular matrix (ECM) in the intervertebral space which results in vertebral fusion. The fusion of the cervical vertebrae observed in the mutant mouse was progressive in nature. Ectopic mineralization of the ECM of the cartilage which would otherwise normally separate carpal bones were noted in the mutant mouse. This lead to the fusion of the carpal bones. Additionally, the mutant mouse was
also noted to have progressive abnormal mineralization of the chondrocostal cartilage of the rib and sterna. Furthermore, the vertebral anomalies in SCT is rather a defect in the boundary maintenance of somites, after the somites themselves have been correctly formed. All the features observed in the mutant mouse were overlapping with features noted in patients with SCT. However, the latter feature has not been observed in patients with SCT.

The variants identified in this study and the earlier reports are all truncating variants [Krakow et al., 2004; Farrington-Rock et al., 2006; Mitter et al., 2008; Yang et al., 2017]. Analysis of truncating variants in FLNB in patients with SCT by an earlier report proved that it results in the loss of filamin B [Farrington-Rock et al., 2006]. A recent study has analyzed the effect of variant, p.S2542Lfs*82 in the HEK293T cell line and demonstrated that it disrupted normal dimer formation [Yang et al., 2017]. Furthermore, null FLNB variant generated in the mouse has essentially the same phenotypic effect as that of SCT which is caused due to deleterious variants in human [Farrington-Rock et al., 2006; Zieba et al., 2016; Lu et al., 2007] which includes short stature, scoliosis, fusion of carpal bones, fusion of sternum and vertebral fusion throughout the entire spine (cervical, thoracic and lumbar) except sacrum which showed normal fusion and no fusion of the caudal region. Mutant mouse also showed abnormal mineralization in the vertebral arches resulting in unilateral or bilateral fusion of vertebrae.

Therefore, loss of FLNB protein due to the presence of truncating variants has been proposed as the mechanism for disease pathogenesis [Farrington-Rock et al., 2006]. All the truncating variants from our study and the earlier reports are distributed across the filamin B protein reinforce that occurrence of deleterious variants at random location
with no protein domain specificity in filamin B underlies the cause of SCT. The absence of molecularly diagnosed SCT phenotype in the heterozygous individuals indicates that none of the variants characterized hitherto acts through haploinsufficiency of filamin B or in a dominant negative manner. A recent study has described an individual with heterozygous variant manifesting short stature and unilateral hip dysplasia [Mitter et al., 2008]. However, they hypothesize that it could be either a non-specific finding or heterozygote manifesting SCT.

Two families have been reported with autosomal dominant SCT due to variants in *MYH3* [Carapito et al., 2016]. Patients from one family had pure SCT, with scoliosis, lumbar lordosis, block vertebrae, short neck with limited mobility, limited extension of both elbows, cleft palate, carpal bone abnormalities which included trapezium-scaphoid coalition, lunate-triquetral coalition and capitate-hamate coalition in one patient whereas carpal bone age delay with no carpal fusion, scaphoid- cuneiform coalition of tarsal bones, motor milestone delay and recurrent otitis media. Patients from second family with variant in *MYH3* displayed intrafamilial variability and had additional features like joint limitation with pterygia and cardiac disease. Evidence of an autosomal recessive SCT due to biallelic variants in *MYH3* have also been reported [Cameron-Christie et al., 2018]. However further clinical and molecular information would be required to describe *MYH3*-related SCT.

### 6.5. Limitations

- Sanger sequencing of genes known to cause MVSD and exome sequencing was carried out for the identification of pathogenic variants because it is cost effective and suitable approach for our center. However, whole genome sequencing may be
a better alternative approach to study MVSD that would also detect copy number variations and variants in the regulatory or intronic regions and novel genes.

- Pathomechanism of the identified variants was not explored via *in vitro* or *in vivo* studies.

**6.6. Future directions**

- With the emerging clinical utility of whole genome sequencing, it would be possible to uncover the complex genetic architecture in a proportion of patients with MVSD.
- A large scale population or epidemiological approach would be useful because some MVSD may be due to teratogens or environmental in origin.
- Some MVSD cases may be multifactorial where genome wide association studies would be useful.
- It remains to be seen if there is a recognizable radiographic appearance for MVSD associated with genes other than *DLL3* and *FLNB*.
- Because very few pathogenic variants have been reported in patients with SCD, more studies on MVSD would help to narrow down the clinical diagnosis for further molecular analysis.