Chapter 6

Discussion
In the 121 unrelated families analyzed, we identified pathogenic mutations in
*WISP3* in majority of them (98 families) followed by *MMP2* variants in 15 families.
The clinical and the mutational spectrums of these conditions, along with the other
inherited arthropathies observed in the cohort are discussed below.

### 6.1 Progressive pseudorheumatoid dysplasia

We studied the clinical and the molecular spectrums of 118 individuals affected with
progressive pseudorheumatoid dysplasia from 98 families.

Majority of the affected individuals in our cohort display classical phenotype of
progressive pseudorheumatoid dysplasia. All the patients were normal at birth
without any signs and symptoms. The general age of onset of symptoms is between
three to six years [15] whereas in a few instances it occurred in adolescence too [14].
The age of onset of PPD in our study varies between 9 months to 16 years with a
mean age of 5.4 years. However, the mean age of diagnosis is 12.7 years. This
difference between the age of onset and diagnosis, suggests the need of awareness
[15].

The adult height in most of the instances is less than two standard deviations below
the mean. Initial signs of the disorder in majority of the patients were gait
abnormalities and muscular weakness leading to easy fatigability. These early signs
were followed by joint swellings and joint pain, which starts in small joints of hands,
gradually developing in all the major joints like knees, hip, wrists, and elbows. Flexion
and extension contractures of joints lead to restricted mobility. The joint stiffness
was another manifestation that progressed like other joint deformities observed in
this disorder. The common knee deformities were demonstrated as genu varum or
genu valgum. The most frequently affected joints were small joints of hands followed
by elbows, knees, hips, and wrists. Joints of feet, ankles and shoulders showed occasional involvement. Decreased mobility of the neck was observed in a few patients. The joint deformities in hands were first noted in proximal interphalangeal joints followed by distal interphalangeal joints. Though, coarse facial features were not a feature of progressive pseudorheumatoid dysplasia, in a few affected individuals the dysmorphic facial features such as triangular face, prominent forehead, midface hypoplasia, upslanted palpebral fissures, dysmorphic nasal bridge, bulbous tip of nose, and short neck were observed. This observation can be subjective and was not significant to propose coarse face as a feature of PPD.

Radiographic evaluation of spine in affected individuals showed typical features of PPD. Irregularities in the ossification of vertebral end plates and flattened vertebral bodies are observed in all the patients with PPD. Platypondyly associated with anterior beaking was a prominent feature. Scoliosis and/or kyphosis were noted often whereas lumbar lordosis was observed very rarely. Loss or narrowing of intervertebral disc spaces was also noticeable in the spine radiographs. The main features observed in radiographs of pelvis were enlarged and flattened capital femoral head along with short and wide femoral neck. The joint deformities in the hips lead to coxa vara. Broadened ilia and irregular acetabular roofs were also observed. The radiographs of other joints showed enlarged epiphyses, widened metaphyses, loss, or narrow joint spaces, irregular articular surfaces, and diffused osteoporosis. Small joints of hands and feet showed proximal interphalangeal joint swellings in early infancy followed by distal joint abnormalities.

A few affected individuals demonstrated atypical phenotype of PPD. Patient numbers 28 and 29 were presented with unusual findings as separation of epiphysis and metaphysis at the upper ends of femur with coxa vara. Both these affected individuals
were siblings. Patient number 26 showed corner fractures at knee joint. Patient number 101 also exhibited slippage of capital femoral epiphysis along with bilateral coxa vara. All other features in these patients were classical PPD findings. Patient number 60 showed atypical phenotypic features such as bowing of both arms leading to rhizomelia in upper limbs. Rest of the physical findings were characteristic of PPD. He had severe disproportionate short stature, (height 124 cm, 6SD below the mean). The radiological examination of the upper limbs revealed bilateral bent, short, and severely deformed humeri with dislocation at the shoulder joint. His younger brother aged 13 years also had clinical features of PPD and right sided rhizomelia.

Until date, nearly 65 mutations have been reported from approximately 185 families in the literature. Of these 17 mutations were contributed from our study. Including the unpublished data, 21 novel mutations were identified in our cohort. These pathogenic sequence variations have been reported in all the exons, except exon 1. Majority of the variants observed were frame shift variants. The variations in the nucleotide sequences such as deletions, insertions, and indels result in the shift of the frame and leads to the formation of a truncated nonfunctional protein. Missense mutations, splice site variants, and nonsense mutations were also observed. The nonsense mutations either by nature or by insertions or deletions leading to shift of the frame constitute 56% (14/25) of the pathogenic variations.

For the novel missense mutations observed in WISP3 gene, the protein modeling studies provided the supporting evidence of pathogenicity. The mutated amino acids resulted in some clashes when compared with the wild type amino acids and most of them exhibited loss of hydrogen bonds. The reference amino acids at the 268 and 337 position are Cysteine. These are known to be involved in disulfide bridge formation (C268-C305; C299-C337). The substitutions at these positions lead to dissociation of
these disulfide bridges. The disruption of these stabilizing disulfide bonds might lead to pathogenic alterations in the structure.

Eight distinct mutations of cysteine residues were reported in this study, of which five were novel variants. The most common mutation observed was p.(Cys337Tyr) with an allele frequency of 28.8% followed by p.(Cys78Tyr) (23.3%) and p.(Cys52*) (13.6%). These variants were observed in high frequency in our cohort. The mutation p.(Cys337Tyr) is specific to the south Indian population and the variant, p.(Cys78Tyr) was found in Bengali and Odisha populations. This indicates that these mutations are specific to Indian population. Various reports in the literature show a few mutations that are specific to some ethnic populations [12]. The genotyping studies for the variants p.(Cys337Tyr) and p.(Cys78Tyr) ascertained the founder effect.

Though the patients 28, 29 and 101 (families 20 and 82) were showed the same atypical features, the pathogenic variants observed in them were different [p.(Cys337Tyr) and p.(Gln269Asnfs*44) respectively]. The affected individuals with the same pathogenic variant do not show the same degree of severity. Variability in the expression of the disorder has been observed with features like, the age of onset, and other important features such as joint swellings, joint contractures, and enlarged femoral epiphyses but are not significant. The zygosity of the mutations also have not shown any correlation. The presence of same mutation in homozygous or in heterozygous state (compound heterozygous) do not result in any detailed changes in the phenotypic expression of the disorder. These observations reflect our earlier observation that there is no specific association between the mutation and the phenotypic features.
6.2 Multicentric osteolysis, nodulosis and arthropathy

Eighteen patients with multicentric osteolysis nodulosis and arthropathy from fifteen families were evaluated in this study. Most of the affected children were apparently normal at birth except in a few instances. The initial symptoms of arthropathy in early childhood were joint pain, contractures or swelling of hands and feet. Small joints of the hands and feet were the most obvious sites of involvement. The progressive destruction of underlying bones lead to pain, swelling and flexion contractures. Knee joints showed swelling and contractures in majority but hip involvement was less severe. Coarsening of facial features, bulbous nose, corneal opacities, strabismus, gingival hypertrophy, and hyperpigmentation of skin were been observed frequently. Subcutaneous, firm, palpable nodules were noted in majority of the affected individuals in the palms and soles. Other cutaneous manifestations including hyperpigmentation and thickening were noted. Serpiginous hyperpigmented cutaneous lesions were present in one patient. The similar phenotypic feature was noted by Zankl et al. and described that it might be elastosis perforans serpiginosa, but was not confirmed [45]. These serpiginous lesions may be caused by the occurrence of abnormal connective tissue elements due to the mutated protein activity.

Most of the affected individuals in the literature were noted with growth restriction (70%) [65]. However, short stature was not a common feature in the present cohort. Cardiac anomalies were observed often in the patients with MONA [52, 53, 65]. The study by Cheng et al. observed an increase in the circulating MMP2 activity in patients with ventricular septal defects [144]. However, the MONA patients with congenital heart defects demonstrate loss of MMP2 enzyme activity [65]. In addition, the studies on mouse and chick revealed the association of MMP2 mutations with heart
development [145, 146]. Taking all these studies into consideration, though the precise role of MMP2 in organogenesis of heart cannot be established but an association of MMP2 activity during formation of the cardia can be suggested.

Radiographs of patients with MONA show marked changes in carpal and tarsal bones with progressive osteolysis and diffuse osteopenia. The thin cortices and osteopenia of medulla are usually seen in all bones as the disease progresses. Gradually, all the carpals are destroyed. Metacarpals and phalanges show similar changes and tend to become broad, irregular with poor remodeling in the later stage of the disease. Low bone density and osteoporosis lead to the fractures in bone and spine [63, 64]. Recurrent fractures were observed in one patient of our cohort.

Most children were apparently normal at birth. The disease generally presented in early childhood with arthropathy. Joint pain, contractures or swelling of hands and feet were symptoms of this serious arthropathy. Some patients presented with joint laxity due to lysis of underlying bones of hands and feet. Fever was notably absent in these children. They demonstrated variable coarsening of facial features, gum hypertrophy, and progressive arthropathy during the course of the disease. Subcutaneous, firm, palpable nodules developed in most of these patients mainly on plantar surface. Their number and size slowly increased with age. Cardiac anomalies were seen in 23% and probably need to be sought by echocardiogram in all the patients with a MMP2 mutation.

Radiographically, lysis of carpal and tarsal bones was observed. The destruction often extended to other bones of hands and feet (metacarpals, metatarsals, and phalanges). The modeling of these bones was defective and they appeared broad and irregular. Osteopenia or osteoporosis was seen in entire skeletal system in older children. Clinically, osteolysis of carpal and tarsal bones, joint disease (contracture,
pain, and swelling) and osteopenia appeared to be the essential features to define the condition, though early in the disease, only osteolysis and osteopenia were apparent. With the disease progression, coarse facial features, hirsutism, and subcutaneous nodules developed. Presence of gum hypertrophy and cardiac anomalies were frequently seen in this condition.

Majority of the mutations were present in exon 8 (30%) and exon 2 (30%). Remaining mutations accumulated in exon 5 (20%), 3 (10%), and 6 (10%). Missense mutations are the most common mutations observed in \textit{MMP2} gene [53]. In our cohort, 50% of the mutations observed were missense variants. Nonsense mutations and deletions, leading to truncated protein were observed in the other 50% of study population. In our study, we have identified a deletion, \texttt{p.(Asn430Thrfs*68)} in 5 patients from 4 families of the same ethnic origin. Along with this, we have identified the previously reported variations, \texttt{p.Arg101Cys}, and \texttt{p.Arg101His} in two families each, making these three variations, as the most common mutations identified in MONA syndrome patients.

Of the novel variants observed in this study, two were missense mutations \texttt{p.(Asp180Val)} and \texttt{p.(Gly410Val)}. These mutations were observed in the position, where a different amino acid substitution was already reported in the literature \texttt{[p.(Asp180Glu), p.(Asp180Asn) and p.Gly410Arg]} [53, 64, 66]. The variant, \texttt{p.(Asp180Val)} was present in a highly conserved position and involved in zinc-ion binding. The substitution of this amino acid with a smaller and neutral amino acid valine might result in loss of contact with zinc ion and hydrogen bonds with other amino acids. The wild type variant, G410 is present in a tight turn of the long helix. However, the mutated amino acid, valine is not as flexible as glycine and the size difference might result in confirmation changes.
Though the overall phenotype appears to be uniform in most of the families with MMP2 mutation, intrafamilial variability and the variations in the expressivity were observed [53]. However, this variable expression cannot be explained by the mutations and their locations. Subcutaneous nodules were not seen in some cases and cannot be explained with the help of genotype. Hirsutism was age dependent and its association with mutation type or location could not be found. Gum hypertrophy (18%) was also rarely observed and was seen in a few patients with truncating mutations located in catalytic the domain. However, it was not consistently observed in all the patients with truncating mutations located in catalytic domain. In the literature, gingival hypertrophy was noted in patients with missense variants and also in patients with mutations in hemopexin domain [52, 61, 64]. Thus, there is no specific association with mutation type and location. Similarly, cardiac anomalies were also not associated with the location of mutation or type of mutation. They were observed in patients with missense mutations in cysteine-switch motif and hemopexin domain [52, 65]. Subcutaneous nodules were present in all the families except four with mutations p.(Glu231*), p.(Val400Argfs), p.Glu404Lys and p.Gly406Asp (our study) [45, 47, 63]. From this, Jeong et al. inferred that all the homozygous mutations present in the catalytic domain are not associated with subcutaneous nodules. In contrast, the catalytic domain mutations identified later by Azzollini et al., and our study showed the association with subcutaneous nodules.

Establishing the genotype phenotype correlation in MONA cases has been challenging because of the following reasons: 1) Limited sample size with mutations noted only in a few domains. 2) The presence of private mutations in almost all the cases being a constrain for statistical calculations. 3) Though there is a variable expression of clinical features like coarse facies, gum hypertrophy, subcutaneous
nodules, and cardiac abnormalities, remarkable phenotypic similarity of radiological features was noted. 4) The same mutation in a family presented inconsistency in the phenotype leading to intrafamilial variability [52, 53, 62]. More number of cases may further help in establishing a genotype-phenotype correlation of MMP2 related disorders.

6.3 Hyaline fibromatosis syndrome

Four affected individuals with HFS were analyzed in this study. Three of the families were noted with consanguinity. All the patients presented with joint contractures and cutaneous lesions. Of these, two patients were with severe HSF and two patients were moderately affected as per the standard grading system [70]. The first two cases with severe phenotype showed the internal organ involvement along with persistent diarrhea, thus classified as severe HSF.

Of the four pathogenic variants observed in HSF patients, the variant c.1074delT (Patient 2) was the common mutation for HSF. We also found a novel mutation, c.1069delG in the hotspot region of ANTXR2 gene in patient 4. The patient 3 was noted with the missense mutation, c.1A>G, at the translation initiation codon, p.(Met1?). This missense substitution at the translation initiation site may lead to no protein production or shifting of the translation initiation site either upstream or downstream. A similar mutation [p.(Met1Arg); c.2T>G] was observed at the same position in another patient with HFS by Antaya et al. [147]. The splice site variant observed in the patient 1 was predicted to be pathogenic with the help of various prediction algorithms.

As per the genotype, the phenotype correlation proposed by Hanks et al. suggests the missense mutations in cytoplasmic domain exhibit a milder phenotype. However,
patients 2 and 4 were noted with frame shift mutations in cytoplasmic tail. Patient 2 exhibited a severe HFS whereas patient 4 demonstrated a moderate phenotype.

6.4 Hyperphosphatemic familial tumoral calcinosis
Two patients with FHTC were evaluated in this study. Both presented with elevated serum phosphate levels. Ectopic calcifications were noted in one patient whereas, hyperostosis was observed in the second. Due to the genetic heterogeneity of this condition, we identified mutations in two different genes in both the patients. In the first family with non-consanguinity, compound heterozygous state was observed in GALNT3 gene. We identified a known missense and a novel nonsense mutation, which has been predicted to be pathogenic. The missense mutation, p.(Leu366Arg) was already reported in an Indian family [148] in a compound heterozygous condition. In the second family with consanguinity, homozygous mutation was noted in FGF23 gene. A known missense, p.Ser129Pro mutation, was observed. This mutation was previously reported in a Japanese family [149]. Another variant, p.Ser129Phe was also reported at the same amino acid position, suggesting the importance of the serine at this particular position [150].

6.5 Uncharacterized arthropathies
6.5.1: A novel multiple joint dislocation syndrome:
The affected siblings clinically showed some resemblance to both SEMD-JL1 and -JL2. However, other features such as airway obstruction due to laryngeal stenosis, facial dysmorphism, kyphoscoliosis at birth, talipes equinovarus, cleft palate and congenital heart disease were absent Thus, they most likely presented with a new type of autosomal recessive SEMD with laxity and joint dislocations. Consanguinity
of the parents suggested a variant affecting function within a shared region of homozygosity (ROH). SNP array revealed ten ROHs in the siblings. However, no causative genes were identified in these regions.

*EXOC6B* is widely expressed in human tissues, such as bone and central nervous system (https://www.proteomicsdb.org/proteomicsdb/#human/proteinDetails/Q9Y2D4/expression). The EXOC6B is a component of the exocyst complex required for tethering secretory vesicles to the plasma membrane and it likely plays a role in polarized exocytosis [151]. Tethering and fusion of exocytic vesicles is required for ciliogenesis which is important for skeletal development [152]. A postulated role of KIF22 in cartilage biology by implication in intracellular transport and/or cilia-associated transport mechanisms [153, 154] indicates a possible link between kinesins (e.g. KIF22) and the exocyst complex (e.g. EXOC6B). In line with this, *NIN*, another gene associated with an SEMD-JL2-like phenotype [155], encodes Ninein required for centrosome maturation and architecture [156]. As the building and the maintenance of a cilium depend on membrane vesicle trafficking, microtubule-dependent motor proteins and formation of basal bodies [157], we speculated that dysfunction of EXOC6B, KIF22 and ninein negatively affects ciliogenesis leading to overlapping SEMD forms. The nonsense variant, p.(Tyr302*) in *EXOC6B* gene leads to loss of the last 509 amino acids of the encoded protein or nonsense-mediated mRNA decay thus making it the most probable causative gene.

**6.5.2: Autosomal recessive skeletal dysplasia with platyspondyly and dysostosis multiplex:**

The siblings’ clinical features were not suggestive of any known skeletal dysplasia and exome sequencing suggested a pathogenic variant in *ARSK* might be the genetic cause for the clinical features observed in this family. Aryl sulfatase K, a member of
sulfatases family of enzymes, is ubiquitously expressed and functions within lysosomes [158]. Sulfatases specifically hydrolyse sulfate esters in glycosaminoglycans, sulfolipids, or steroid sulfates, thereby playing key roles in cellular degradation, cell signalling, and hormone regulation. This enzyme is highly conserved among various vertebrates and invertebrates suggesting an important role in cellular function [159]. Other human diseases with skeletal dysplasia already known to be associated with pathogenic variations in sulfatases include, chondrodysplasia punctata type 1 (arylsulfatase E), mucopolysaccharidosis type 2 (iduronate-2-sulfatase), mucopolysaccharidosis type 3A (sulfamidase), mucopolysaccharidosis type 3D (glucosamine-6-sulfatase), mucopolysaccharidosis type 4A (galactosamine-6-sulfatase) and mucopolysaccharidosis type 6 (arylsulfatase B). Owing to the similarity of biological functioning and phenotypes associated with deficiency of these enzymes, these findings implicated that the mutations in ARSK might cause a skeletal dysplasia with platyspondyly and dysostosis multiplex.

6.6 Limitations of the study

- The study population comprised only common inherited arthropathies: progressive pseudorheumatoid dysplasia, multicentric osteolysis nodulosis and arthropathy, hyaline fibromatosis syndrome and hyperphosphatemic familial tumoral calcinosis and not rarely referred arthropathies.
- Clinical and radiological details were incomplete in a few affected individuals.
- As majority of the patients with multicentric osteolysis nodulosis and arthropathy were from referral centers, the unavailability of fresh blood samples hindered the study of MMP2 enzyme activity.
• The genotype phenotype correlation could not be assessed due to the small sample size for the conditions like hyaline fibromatosis syndrome and hyperphosphatemic familial tumoral calcinosis.

6.7 Future directions

• Studies focused on understanding the pathophysiology of arthropathies will assist in developing therapeutic approaches.

• Functional characterization of the novel variants identified in this study.

• Delineation of novel phenotypes of monogenic arthropathies. For the inherited arthropathies with unknown genetic aetiology, next generation sequencing based testing strategies could be used in identifying the genetic basis.