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Summary of the thesis

The aim of the doctoral thesis is to present the reader with the research of clubfoot deformity, specifically the relapsed clubfoot, and establish clubfoot fibrotic tissue in the biomechanical context. The thesis is organized in a book format, and each chapter is dedicated to a coherent topic. The topic of each chapter is consequently specified for the relapsed clubfoot and the researched fibrotic tissue. The final chapter discusses and concludes on the conducted research described in the thesis and specifies the limitations of the research and possibilities for further research.

Chapter I

Chapter I introduces the clubfoot deformity. Macro-morphology, phenotype and aetiology of clubfoot are briefly described. The clubfoot is established in the context of fibroproliferative disorders, e.g., Dupuytren's contracture or Peyronie's disease. The main characteristic of fibroproliferative disorders is fibrosis, a process of excess collagen deposition by cells. Conservative treatment includes the Ponseti method of mechanical manipulation and casting or French functional therapy for muscle stimulation. However, clubfoot can attain various degrees of severity. The severity of the deformity is determined based on the Pirani or Dimeglio classification method. In severe cases where the clubfoot relapsed after conservative treatment, the orthopaedic practitioners adopt invasive surgical treatment. The studied fibrotic tissue and control samples were extracted in surgical operations of relapsed clubfoot. The characterization of the patients is presented in Table 1

of the thesis. The fibrous tissue and controls extracted during surgical treatment were used in the research presented in the following chapters of the thesis.

Chapter II

Chapter II navigates the techniques of sample preparation and presents several limitations of the preparation of the sample in terms of altering mechanical properties. The chapter is introduced with an overview of the sample manipulation techniques. All extracted tissue decays; therefore, the tissue must be fixed to prevent or delay the decay. The chemical fixation was unsuitable for fixing the relapsed clubfoot biopsies, and freezing at −80 °C was adopted to maintain the samples' integrity. The clubfoot samples were later prepared for morphological and biomechanical characterization. In order to access deeper layers of the tissue, the samples were dissected at Cryostat into 120 μm thick sections. The sections were compatible with optical microscopy for morphological analysis. However, the samples were not stained. Staining the samples with fluorescent dyes could alter the micro-mechanical properties of the tissue sections. Therefore, the morphological characterization was performed with label-free optical microscopy. Further tissue sample processing included ablation for identifying the same structures at different microscopy sites. The ablation created a rectangular mark, an ablation crater in the collagenous tissue. The chapter finishes with the problem of dehydration and rehydration of the relapsed clubfoot tissue.

Rehydration was required to preserve the samples and fix the tissue on the microscopy slide for atomic force microscopy (AFM). The hydration level is described to affect the change of tissue mechanical properties, and the samples presented in the thesis were left to hydrate for 30 minutes.

Chapter III

Chapter III advances the description of the clubfoot fibrotic tissue, its morphology and visualization. The chapter describes the visualization techniques used in visualizing the relapsed clubfoot tissue. The visualization techniques are compatible with the mechanical testing of the tissue without introducing artifacts. The chapter contains results of the morphological characterization of the relapsed clubfoot tissue. The first described label-free method is second harmonic generation (SHG), which was used for identifying intact collagen regions in the relapsed clubfoot tissue sections at the primary microscopy site. The analysis of SHG images revealed that the tissue from the medial side of a relapsed clubfoot contains more SHG signal per FOV than the lateral side. Therefore, the conclusion is that the medial side contains more collagen than the lateral side. SHG was also used as a top-down projection (TPD) in the analysis of the surface roughness, which cannot exceed 15 μm for an AFM force map. TPD projects the first detected pixels with SHG signal and its depth information into a single image as it evaluates the Z-stack planes from top to bottom plane. The second label-free method used in the characterization of the relapsed clubfoot tissue morphology was polarization light microscopy (PLM). The PLM is based on the collagen's birefringence, which produces extraordinary waves of polarized light that can be filtered and selectively detected. The PLM is used at the secondary

microscopy site and helps navigate the field of view to an area previously visualized by SHG. The analysis of PLM images was concluded with the significant result of collagen crimp pattern propagating at a higher frequency in medial side tissue than in lateral side tissue. The third label-free method was third harmonic generation (THG), which was used to identify adipose tissue. The THG identifies areas with high refractive index differences, such as the water-lipid interface. The medial side tissue had fewer areas with THG signal per FOV compared to the lateral side tissue. The chapter also identifies a relationship between clubfoot morphology and the mechanical properties of fibrotic tissue.

Chapter IV

Chapter IV is dedicated to the problems of viscoelastic materials and establishes the clubfoot fibrotic tissue in the context of biomechanics. AFM is presented as the biomechanical test of choice for characterizing the mechanical properties of the relapsed clubfoot tissue. The reasoning for selecting the AFM is clarified by the physical size of the samples, which are small for conventional tensile testing. AFM is an indentation-based method with a small probe mounted on a spring cantilever. A crucial part of AFM measurements is the immobilization of the sample to measure mechanical properties correctly and avoid movement artifacts. However, some sample immobilization techniques, such as gluing the tissue to the microscopy slide, could alter sample properties. The sample preparation methods of freezing and dehydration, described in Chapter II, played a vital role in adhering the relapsed clubfoot section to the microscopy slide. With the immobilized and rehydrated sample,

the AFM was measured in force spectroscopy mode, and the theoretical model was used to calculate the Young's moduli of the tissue. The selected theoretical model for fitting the force-distance curves was the Hertz-Sneddon model. Afterwards, the relapsed clubfoot tissue is compared to other tissue measured with AFM in literature to create a sense of scale for the mechanical properties detectable by the method. Finally, the results of the AFM indentation are presented in the chapter. The medial side tissue has higher values of Young's modulus than the lateral side tissue. The data from AFM and SHG suggest that the excess collagen production contributes to observed stiffness in medial side tissue by orthopaedic practitioners. However, AFM data suggest that a micro-structural change in the medial side tissue also contributes to the overall stiffness of the tissue.

Chapter V

Chapter V brings the measured morphological data and data of the mechanical properties together in multimodal analysis. Multimodal analysis was performed by co-registering the SHG images, PLM images, AFM brightfield images and AFM force maps based on the ablated rectangular mark. The summary of morphological and mechanical characterization is presented. The relapsed clubfoot fibrotic tissue of the medial side is compared with the connective tissue of the lateral side clubfoot in terms of mechanical properties, collagen and adipose tissue content, and fibre orientation. The chapter highlights the methodological importance of correlative microscopy and reveals that the rehydration between different microscopy sites has modified the shape of the tissue.

Chapter VI

Chapter VI focused on the limitations of the study, and the results are discussed in terms of the methodology and statistics. The chapter concludes on the results presented in the thesis.

List of Publications

The conducted research presented in the thesis was published in international journals. Six abstracts of the publications are presented in the thesis, four of which were published on the topic of relapsed clubfoot deformity with biological relevance and two additional publications were published with methodologically relevant topics to the presented research work.

Bibliography (78)

There are 224 cited works, which include both scientific papers and books.

Intellectual value of the thesis

The conducted research presented in the thesis is the first talipes equinovarus congenitus research quantifying the mechanical properties of the relapsed clubfoot tissue. The medical field is performing fewer surgical treatments of relapsed clubfoot, and tissue research will prove far more difficult in the future with the lack of extracted tissue. The work may as well be one of the last that quantifies the mechanical properties of the relapsed clubfoot tissue as the conservative medical treatment is well equipped to tackle the overall treatment of the clubfoot.

However, the work performed in the thesis to achieve results highlights the necessity for multimodal analysis, as multimodal analysis investigates biological tissue from multiple points of interest. Automation of the evaluation process is indispensable to evaluate the large data samples produced from different modalities. Automating the evaluation process allows us to bypass the human error of accidentally misinterpreting data. Therefore, the thesis can be a methodological inspiration for fellow students undertaking the research work.