Chapter 6

Imaging mass spectrometry of myxoid sarcomas identifies proteins and lipids specific to tumour type and grade, and reveals biochemical intratumour heterogeneity

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* and ** contributed equally to manuscript

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Abstract

Myxofibrosarcoma and myxoid liposarcomas are relatively common soft tissue tumours that are characterized by their so-called myxoid extracellular matrix and have to some extent overlap in histology. The exact composition and potential role of their myxoid extracellular matrix are insufficiently understood. To gain more insight into the biomolecular content of these tumours we have studied 40 well-documented myxofibrosarcoma and myxoid liposarcoma cases using imaging mass spectrometry. This technique provides a multiplex biomolecular imaging analysis of the tissue, spanning multiple molecular domains and without a priori knowledge of the tissue’s biomolecular content. We have developed experimental protocols for analyzing the peptide, protein and lipid content of myxofibrosarcoma and myxoid liposarcomas, and have detected proteins and lipids that are tumour-type and tumour-grade specific. In particular lipid changes observed in myxoid liposarcomas could be related to pathways known to be affected during tumour progression. Unsupervised clustering of the biomolecular signatures was able to classify myxofibrosarcoma and myxoid liposarcomas according to tumour type and tumour grade. Closer examination of histologically similar regions in the tissues revealed intratumour heterogeneity, which was a consistent feature in each of the myxofibrosarcoma studied. In intermediate grade myxofibrosarcoma it was found that single tissue sections could contain regions with biomolecular profiles similar to high-grade and low-grade tumours, and that these regions were associated with the tumour's nodular structure, thus supporting a concept of tumour progression through clonal selection.
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Introduction

Myxoid tumours of soft tissue are a heterogeneous group of mesenchymal origin and characterized by their so-called myxoid extracellular matrix (ECM)[1,2]. In this group, myxofibrosarcoma and myxoid liposarcoma are relatively common, mostly occurring in the extremities. Although their histology overlaps to some extent, these tumours have different clinical behaviour[3]. Recently, we demonstrated that the composition of the myxoid ECM is tumour type and tumour grade dependent by conventional histochemical techniques as well as by mass spectrometry-based proteomics[3,4]. We illustrated that these differences in ECM composition paralleled the different biological and clinical behaviour of these tumours[4]. Myxofibrosarcoma is characterized by non-specific cytogenetic aberrations that increase with grade, suggesting a multistep tumour progression model due to acquired genetic instability[5]. Low-grade myxofibrosarcoma is microscopically characterized by a multinodular growth pattern[6]. Most human malignancies originate from a single cell but display intratumour heterogeneity by the time of diagnosis. This heterogeneity might be due to metabolic and epigenetic differences in the tumour though strong evidence showed that intratumour heterogeneity also arises from genetically distinct clones within the tumour[7-13]. This suggests that these tumours progress through multiple nodules, one of which becomes more dominant and will progress towards high-grade myxofibrosarcoma, as has been shown in other neoplasms[14-16]. How this clonal selection is reflected at the level of protein expression is still unclear. Furthermore, methods for the combined analysis of phenotypic and protein diversity at microscopical level in-situ in tissue sections are also lacking. In contrast to myxofibrosarcoma, myxoid liposarcoma is characterized by its predominant tumour specific translocation t(12;16) (q13;p11) involving the FUS and DDIT3 genes, whose transcribed fusion protein acts as a transcription factor[17-20]. This transcription factor plays a pivotal role in its tumourigenesis by regulating the expression of genes involved in cell proliferation[20-22]. It also controls the transcription of C/EBP which affects the expression of peroxisome proliferator-activated receptor gamma, a key player in adipocytic differentiation[19,23,24]. This links the FUS/DDIT3 fusion protein to its histological phenotype. To get more insight into changes in the expression of peptides, proteins and lipids during tumour progression we studied a well documented series of myxoid liposarcoma and myxofibrosarcoma tissues using MALDI imaging mass spectrometry. This rapidly developing technique uses spatially resolved proteomic and metabolomic methods to simultaneously trace the distributions of hundreds of peptides, proteins, or metabolites in a tissue section[25]. The technique uses the masses of the biomolecules to distinguish between different species and thus does not require any form of labelling. The biomolecular profiles can be used to obtain biomolecular signatures associated with specific histological features, to distinguish different regions within a tissue or to differentiate and classify tissues and is beginning to have in impact in cancer research[26-31]. In the present study we
investigated the ECM of myxofibrosarcoma and myxoid liposarcoma by using MALDI imaging mass spectrometry to analyze the peptide, protein and lipid content of low- and high-grade myxofibrosarcoma and low- and high-grade myxoid liposarcoma. This allowed a detailed examination of the myxoid ECM of these tumours, especially in relation to their grade specific phenotype and indicated that the technique may also reveal the intertumoural and intratumoural biomolecular heterogeneity of histologically contiguous tumours.

**Material and Methods**

*Tissue/clinicopathological data*

Slides were re-evaluated histologically and classified according to the 2002 World Health Organization criteria[32,33]. Myxofibrosarcoma cases were histologically graded according to the French Fédération Nationale des Centres de Lutte Contre le Cancer[34]. Myxoid liposarcoma cases were graded on the basis of percentage of their round cell component[32,33]. All tissue samples were handled in a coded fashion, according to Dutch national ethical guidelines (Code for proper secondary use of human tissue, Dutch Federation of Medical Scientific Societies).

*Sample preparation*

Tumour tissue samples obtained from surgical resection specimens were snap frozen in liquid isopentane and then stored at -80 °C until sectioning. 5 μm thick tissue sections were cut at -20 °C using a cryomicrotome and stained with hematoxylin & eosin (H&E) to check diagnosis and viability of the tissue. For the MALDI imaging mass spectrometry experiments 12 μm thick tissue sections were cut at -20 °C and thaw mounted onto conductive glass slides (Delta Technologies, Stillwater, USA). The tissues were then slowly brought to room temperature in a desiccator and prepared for MALDI analysis of the tissue's peptides, proteins, or lipids.

Protein and peptide imaging: The tissues were washed in ice-cold 70% ethanol (2x30 seconds), dried under a stream of nitrogen and a uniform coating of the MALDI matrix deposited onto the tissue using an ImagePrep device (Bruker, Bremen, Germany) and a 20 mg/ml solution of sinapinic acid in 6:4 AcN:0.5% TFA(aq.).

Peptide and protein profiling: The tissues were washed in ice-cold 70% ethanol (2x30 seconds), dried under a stream of nitrogen and 1 l droplets of the sinapinic acid matrix solution deposited onto the tissue. Lipid imaging: A uniform coating of 2,5-dihydroxybenzoic acid was added using an ImagePrep device and a 10 mg/ml solution in 7:3 MeOH:H2O.

Immediately prior to loading the sample in the mass spectrometer, a 1200 dpi scan was recorded using a flatbed scanner.
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Mass Spectrometry
Peptides and proteins
All peptide and protein mass spectrometry experiments were performed on an Autoflex III mass spectrometer (Bruker Daltonics, Bremen, Germany) and were acquired in fully automated mode using the Flex software suite (FlexControl 3.0, FlexImaging 2.1, FlexAnalysis 3.0, Bruker). Imaging mass spectrometry experiments were performed using 100 µm pixel size, 600 laser shots per pixel (50 laser shots per position of a random walk within each pixel). During definition of the imaging mass spectrometry experiment the dataset is manually aligned with an optical image of the tissue, and were then subsequently aligned with an optical image of the H&E stained tissue (tissue stained after the imaging mass spectrometry experiment[35]. Profiling mass spectrometry experiments were performed by accumulating the signals from 1200 laser shots (50 laser shots per position of a random walk within each matrix spot).

Lipids
Lipid imaging mass spectrometry experiments were performed using an UltraflexXtreme mass spectrometer (Bruker Daltonics, Bremen, Germany) and were acquired in fully automated mode using the Flex software suite (FlexControl 3.3, FlexImaging 2.1, FlexAnalysis 3.3) and used 100 µm pixel size with 500 laser shots per pixel (100 laser shots per position of a random walk within each pixel). Lipid peak assignments were made by first comparing each peak's accurate mass measurement with the LIPID MAPS database (http://lipidmaps.org, mass accuracy 0.005 Da) and then confirming the assignments by MS/MS of selected peaks.

Data Analysis
Each pixel's spectrum was first processed using a smoothing and baseline subtraction routine using FlexAnalysis. A Gaussian smoothing algorithm was used for mass spectral smoothing (proteins: width 2 m/z and 4 cycles; lipids: width 0.05 m/z and 3 cycles) and a ConvexHullV3 algorithm was used for baseline subtraction. Each pixel's or each profile's mass spectrum was then normalized to its total ion count. These mass spectral processing steps have been previously shown to result in superior quantitative capabilities[36,37]. Statistical analysis was performed using ClinProtTools 2.2 and the results visualized in FlexImaging 2.1 (Bruker Daltonics, Bremen, Germany). An automated peak selection algorithm then reduced the dimensionality of the dataset to the 300 most intense peptide and protein peaks.

Unsupervised analysis: Principal component analysis was performed using mean-centering and auto-scaling. Hierarchical clustering was performed by first reducing non-correlated signals in the data by applying a principal component analysis data reduction step[38]. All data that constituted 90% of the variance was retained, the 10% omitted from the analysis was found to contain mostly uncorrelated signals. Hierarchical
clustering was performed using the Euclidian distance between each pixel's mass spectrum and an average linkage. All results of hierarchical clustering or principal component analysis were then presented as false colour images on the tissue sections to examine the spatial variation of the pixels found to have correlated mass spectra, and to compare these results with each tissue's histology.

Supervised analysis: all tissues were first annotated based on a histological analysis of the H&E stained tissues. Mass spectra from multiple patients with low grade MFS, defined as class 1, and spectra from multiple patients with high grade MFS, defined as class 2, were used to build a support vector machine classification algorithm, which was then used to reveal the regions with a low/high grade biomolecular signature in intermediate grade MFS.

To compare and contrast intratumour heterogeneity across multiple patient samples six localized regions within one intermediate grade MFS were selected, based on a histological analysis of the tissue, and used to create a support vector machine model. This SVM classifier was then applied to additional patient samples of intermediate grade MFS, the results presented as false colour images and compared with each tissue's histology.

Results

Clinicopathological data
Clinicopathological data are summarized in table 1. Myxofibrosarcomas and myxoid liposarcomas both showed a slight male predominance. The median age of occurrence was 67.9 years for myxofibrosarcomas (low-grade: 66.4 and 69.4 for high-grade) and 44.9 for myxoid liposarcomas (low-grade: 42.2 and high-grade: 47.6). All tumours occurred in the extremities, except for one low-grade myxofibrosarcoma (case 10: occiput) and 1 high-grade myxoid liposarcoma (case 37: pelvic region). Most cases were primary tumours including a higher number of (non-related) local recurrences and metastasis in both groups of high-grade tumours. All myxofibrosarcomas exhibited increased numerical and structural non-specific cytogenetic aberrations upon increase in grade[4,5]. All myxoid liposarcomas contained the tumour specific t(12;16) translocation as confirmed by FISH and/or RT-PCR (data not shown).

Tissue profiling
Aged tissue samples or tissues that have been in contact with Tissue-Tek optimal cutting temperature polymer can lead to poor MALDI mass spectrometry results[39]. A quality-control experiment was first performed to ensure that tissues included in the subsequent imaging mass spectrometry analysis generated rich peptide and protein profiles. Immediately after mass spectrometry analysis the matrix was removed and the tumours H&E stained[35]. Each tissue was then examined to make sure the tissues did not contain necrotic areas. Tissue sections from ten low-grade myxofibrosarcoma, ten
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Table 1: Clinicopathological data of included myxoid soft tissue tumours

<table>
<thead>
<tr>
<th>Sample</th>
<th>Tumour type</th>
<th>Grade</th>
<th>P/R/M</th>
<th>Age</th>
<th>Gender</th>
<th>Site</th>
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<tbody>
<tr>
<td>1</td>
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<td>P</td>
<td>55</td>
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<td>P</td>
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<td>P</td>
<td>71</td>
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<td>4</td>
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<td>P</td>
<td>63</td>
<td>F</td>
<td>right hamstring muscles</td>
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<td>R</td>
<td>84</td>
<td>M</td>
<td>subcutaneous left lower arm</td>
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<tr>
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<td>P</td>
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<td>P</td>
<td>39</td>
<td>F</td>
<td>subcutaneous occiput</td>
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<td>pretibial superficial</td>
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<td>R</td>
<td>61</td>
<td>M</td>
<td>in left musculus gracilis</td>
</tr>
<tr>
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<td>65</td>
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<td>right lower leg</td>
</tr>
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<td>14</td>
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<td>43</td>
<td>M</td>
<td>in right musculus deltoideus</td>
</tr>
<tr>
<td>15*</td>
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<td>P</td>
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<td>F</td>
<td>left elbow (intramuscular)</td>
</tr>
<tr>
<td>16</td>
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<td>R</td>
<td>57</td>
<td>F</td>
<td>right elbow (intramuscular)</td>
</tr>
<tr>
<td>17</td>
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<td>76</td>
<td>F</td>
<td>in musculus sartorius</td>
</tr>
<tr>
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<td>P</td>
<td>66</td>
<td>M</td>
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<tr>
<td>19</td>
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<td>high grade</td>
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<td>64</td>
<td>F</td>
<td>left lower leg (superficial)</td>
</tr>
<tr>
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<td>71</td>
<td>M</td>
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</tr>
<tr>
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<td>33</td>
<td>M</td>
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<td>54</td>
<td>F</td>
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<tr>
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<td>46</td>
<td>M</td>
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<td>P</td>
<td>29</td>
<td>M</td>
<td>upper leg right, subcutaneous</td>
</tr>
<tr>
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<td>P</td>
<td>62</td>
<td>M</td>
<td>right sole ar muscle</td>
</tr>
<tr>
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<td>R</td>
<td>39</td>
<td>M</td>
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<td>27</td>
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<td>R</td>
<td>42</td>
<td>F</td>
<td>intra-abdominal</td>
</tr>
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<td>41</td>
<td>M</td>
<td>left gastrocnemius muscle</td>
</tr>
<tr>
<td>29*</td>
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<td>R</td>
<td>43</td>
<td>F</td>
<td>right hamstring muscles</td>
</tr>
<tr>
<td>30</td>
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<td>P</td>
<td>33</td>
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<td>32*</td>
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<td>high grade</td>
<td>R</td>
<td>45</td>
<td>M</td>
<td>left hamstring muscles</td>
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<tr>
<td>33</td>
<td>HLS</td>
<td>high grade</td>
<td>P</td>
<td>34</td>
<td>F</td>
<td>right vastus lateralis muscle</td>
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<tr>
<td>34</td>
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<td>high grade</td>
<td>M</td>
<td>52</td>
<td>M</td>
<td>left abdominal muscles</td>
</tr>
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<td>P</td>
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<td>F</td>
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<td>P</td>
<td>45</td>
<td>F</td>
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<td>37</td>
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<td>high grade</td>
<td>P</td>
<td>60</td>
<td>F</td>
<td>pelvis</td>
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<td>M</td>
<td>left hamstring muscles</td>
</tr>
<tr>
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<td>HLS</td>
<td>high grade</td>
<td>M</td>
<td>39</td>
<td>M</td>
<td>right popliteal fossa</td>
</tr>
<tr>
<td>40*</td>
<td>HLS</td>
<td>high grade</td>
<td>R</td>
<td>42</td>
<td>F</td>
<td>right vastus medialis/semimembranosus muscles</td>
</tr>
</tbody>
</table>

Abbreviations: MFS: myxofibrosarcoma; MLS: myxoid liposarcomas; P: primary tumour; R: local recurrence; M: metastasis; M: male; F: female.

Cases with asterisk (*) were included in imaging mass spectrometry experiments.

high-grade myxofibrosarcoma, ten low grade-myxoid liposarcoma, and ten high-grade myxoid liposarcoma patients were analyzed using MALDI-ToF protein profiling. Tissues free of necrosis, which did not exhibit Tissue-Tek contamination and which generated rich peptide and protein profiles were retained. Five tissues from each tumour group were then randomly selected for MALDI imaging mass spectrometry.
Chapter 6

Unsupervised clustering distinguishes myxofibrosarcoma and myxoid liposarcoma according to tumour type and tumour grade

For the profiling experiments approximately 2000 spectra were recorded from each tissue. Random selections of 200 spectra from each tissue were then imported into the statistical analysis software ClinProtTools. Figure 1 shows examples of mass spectra obtained from the different individual tissue sections, and clearly demonstrates that low-grade myxofibrosarcoma, high-grade myxofibrosarcoma, low-grade myxoid liposarcoma and high-grade myxoid liposarcoma generate different peptide and protein profiles, and that each tumour's profile is highly reproducible. An automated feature recognition system was then used to reduce these spectra to the 300 most abundant peaks (each peak corresponds to the mass of a peptide or protein), and principal component analysis performed. This is a multivariate analysis technique frequently used to distinguish underlying trends in highly complex datasets[30]. The results are a series of principal components which describe the largest variance (spread) in the dataset (principal component 1), the next largest variance (principal component 2), and so on. The first three principal components of the myxoid tumour profiling data reveal four specific groupings which differentiate between high-grade myxofibrosarcoma, low-grade myxofibrosarcoma, high-grade myxoid liposarcoma and low-grade myxoid liposarcomas (figure 1). Principal components 1 and 2, which describe the largest variance in the data, discriminate between low-grade myxofibrosarcoma, low-grade myxoid liposarcomas and the high-grade tumours. High-grade myxofibrosarcoma and high-grade myxoid liposarcoma showed considerable overlap in principle component 1 and principal component 2 but were clearly separated in principal component 3. Principal component analysis also provides the contribution of each protein to each principal component and thus revealed which protein peaks discriminate between the different myxoid tumours and grades. This included peaks consistent with proteins previously shown to discriminate between high-grade and low-grade soft-tissue sarcomas including calgizzarin, calcyclin, calgranulin, histone H2A, histone H2B, histone H3 and histone H4 [40].

MALDI imaging mass spectrometry of peptide and proteins in myxoid soft tissue tumours

Tissues sections from the five selected patient samples of high-grade myxofibrosarcoma, low-grade myxofibrosarcoma, high-grade myxoid liposarcoma and low-grade myxoid liposarcoma were prepared for peptide and protein MALDI imaging mass spectrometry and analyzed with a pixel size of 100 m using an Autoflex III MALDI-ToF. Each tissue generated rich datasets describing the distributions of a large number of peptide and protein ions, and included several proteins that were upregulated in specific tumour types and grades. Figure 2 shows examples of tumour specific proteins detected by imaging mass spectrometry and also includes a table listing the masses of multiple tumour and grade specific protein peaks, several of which are consistent with identified
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Figure 1: Initial screening of spectra obtained from the tissues of 10 high-grade and 10 low-grade myxofibrosarcoma (A and C) and 10 high-grade and 10 low-grade myxoid liposarcomas (B and D) was used to select tissues that generate rich high quality peptide/protein spectra (E). F: 3D plotting graph of principal component analysis of these samples separate low-grade tumours in principal component 1 (PC1) and principal component 2 (PC2) and high-grade tumours in principal component 3 (PC3). Corresponding 2D plots of the same analysis were visualized in figure panels G-I. Orange: high-grade myxofibrosarcoma; red: high-grade myxoid liposarcoma; blue: low-grade myxofibrosarcoma; green: low-grade myxoid liposarcoma.

proteins previously revealed found to discriminate between high-grade and low-grade soft-tissue sarcomas (calcylin, m/z 10090; Histone H2A, m/z 14007)[40].

Lipid profiling of myxoid liposarcoma separates low-grade from high-grade tumours MALDI imaging mass spectrometry of myxoid liposarcoma revealed differential lipid profiles according to tumour grade that could be used to classify the tissues and that are consistent with the known molecular pathology of the tumour. A comparison of the lipid signatures obtained from low- and high-grade liposarcomas is shown in figure 3. Low- and high-grade tumours each contain specifically expressed lipids and whose images clearly reveal their localization to one grade. These lipids were then identified using MS/MS and the lipidmaps database. Phospholipids, particularly phosphocholines, were detected in both low and high-grade tumours but were detected at higher levels in
Figure 2: Discriminating power of imaging mass spectrometry illustrated by tumour and grade specific m/z values. The images illustrate the distribution of intensities of tumour type and grade specific m/z values (left panel). Table shows tumour-and grade specific m/z values. Abbreviations: MFS: myxofibrosarcoma; MLS: myxoid liposarcomas: low: low-grade; high: high-grade.

the high-grade tumours. Low-grade myxoid liposarcoma contained additional peaks due to triacylglycerols.

Myxofibrosarcoma shows intratumour heterogeneity
MALDI imaging mass spectrometry enables the variation of biomolecular content within single tissues to be examined and compared with its histology. Macroscopically and microscopically, myxofibrosarcoma is characterized by a multinodular growth pattern[6]. Close examination of low- and intermediate-grade myxofibrosarcoma datasets revealed that different locations within the same tumour generated different peptide/protein spectra and that these differences appear to be related to the nodular structure of the tissue.

Mass spectra from the peptide and protein datasets of high grade, low grade and intermediate grade myxofibrosarcoma samples were analyzed using a hierarchical clustering algorithm. Figure 4A shows that a significant number of the spectra (329) from the intermediate-grade tumour clustered with the high grade myxofibrosarcoma patient samples, and the remaining spectra (706) clustered with the low grade myxofibrosarcoma patient samples. In both instances the intermediate-grade spectra were separated from the high-/low-grade spectra at the next level of hierarchical tree, indicating that the intermediate spectra were high-/low-grade-like but were also quite distinct from the real high-/low-grade tumours.

To investigate this intratumour heterogeneity further the mass spectra from multiple high grade myxofibrosarcoma patient samples and multiple low grade myxofibrosarcoma patient samples were used to build a support vector machine...
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Figure 3: Lipid imaging mass spectrometry of high- and low-grade myxoid liposarcoma reveals differential lipid profiles and the spatial distributions show the localization of specific lipids in one tumour grade. The lipids were identified using tandem mass spectrometry. The experimental mass, the assignment and the mass accuracy of the assignments are provided alongside the image. * matrix clusters.

classification algorithm. Application of this high-grade/low-grade classifier to intermediate grade myxofibrosarcoma samples revealed that different regions of the intermediate grade tissue samples generated biomolecular MS signatures consistent with high-grade or low-grade tumours, and which resembled the characteristic multinodular morphology of myxofibrosarcoma tumours (figure 4B).

Figures 4A and 4B indicate that the intermediate grade myxofibrosarcoma tissue possessed nodules with high-grade- and low-grade-like character, but did not address if the nodules can be further subdivided on the basis of their biochemical signatures nor if the same intratumour heterogeneity is present in multiple tissues. To address these questions six different regions-of-interest were selected in a tissue sample (indicated in figure 4C left panel), based on a histological analysis of the tissue, and a new support vector machine classification model generated. This model was then applied to multiple intermediate grade myxofibrosarcoma patient samples. The results of this analysis demonstrate that many intermediate grade tissues exhibit significant biochemical intratumour heterogeneity, figure 4C. Within the two areas highlighted at low-grade-like and high-grade-like in figure 4B, figure 4C indicates that both areas consist of multiple nodules with different protein signatures.

Discussion

Myxofibrosarcoma and myxoid liposarcoma are, as their names imply, characterized by their abundant so-called myxoid ECM. To study the molecular composition of the ECM of these tumours in relation to tumour progression, we performed imaging mass spectrometry in a well-documented series representing their low-grade and high-grade
variants. The imaging mass spectrometry experiments were performed using matrix assisted laser desorption/ionization (MALDI), an ionization technique that enables highly sensitive mass analysis of biomolecules[41]. MALDI imaging mass spectrometry combines the advantages of biomolecular mass spectrometry, unbiased and simultaneous analysis of multiple biomolecules (peptides, proteins and lipids), with an analysis of their spatial distribution[25,42-44]. Principle component analysis of total m/z spectra showed that myxofibrosarcoma and myxoid liposarcoma clustered separately according to tumour type and tumour grade (figure 1). Based on the principle component analysis and corresponding loading plots, m/z values that contributed most to the separation of each group could be identified. From principal component 1 and principal component 2 it is clear that high-grade myxofibrosarcoma and high grade myxoid liposarcoma show considerable overlap. Low-grade myxofibrosarcoma and low-grade myxoid liposarcoma clustered most separately. In other words, most variance is seen between the two different groups of low-grade tumours compared to the two different groups of high-grade tumours. This means that although both low-grade tumours show some degree of histological overlap (i.e. their abundant myxoid ECM), the biochemical composition of their ECM differed significantly. Imaging mass spectrometry identified tumour type and tumour grade specific peptides, proteins and lipids in the tissue section (figures 2 and 3).

Figure 4: A) Hierarchical clustering analysis of mass spectra from low-(dark blue), intermediate- (light blue) and high-grade (orange) myxofibrosarcoma reveals differential clustering of the intermediate grade spectra into low-grade-like and high-grade-like spectra. B) Application of classification algorithm to an intermediate-grade myxofibrosarcoma tissue, which was developed to distinguish between high-grade and low-grade myxofibrosarcoma tissues using five patient samples of each class, indicates the nodular structure of the low-grade-like and high-grade-like regions of the tissue. C) Six localized regions were selected on the basis of a histological examination of an H&E stained tissue section (indicated) and a classification algorithm developed to distinguish between the different regions. Application of the classification algorithm to multiple intermediate-grade myxofibrosarcoma tissues reveals a nodular like structure in each tissue.
Imaging mass spectrometry of myxoid sarcomas

Myxofibrosarcoma is characterized by multinodular growth[6]. These nodules, especially in low-grade tumours, can be histologically identical. The results reported here, and intimated previously[31,38,45], indicate that imaging mass spectrometry may be used to study intratumour biomolecular heterogeneity of primary human tumours, directly on tissue slides. Despite their histological resemblance imaging mass spectrometry revealed that intratumour heterogeneity was a consistent feature in each of the myxofibrosarcomas studied, and revealed that different nodules exhibited high-grade-/low-grade-like biomolecular signatures (figure 4). Intratumour heterogeneity has been described in many neoplasms, as well in soft tissue sarcomas[13,46]. Orndal et al. have already shown intratumoural cytogenetic variability by identifying karyotypically different though cytogenetically related clones in different parts of the same tumour. Hence, cytogenetic heterogeneity and clonal evolution seem to be common in soft tissue sarcoma and have been detected most often in malignant fibrous histiocytoma and leiomyosarcoma[47]. Similar intratumour heterogeneity was detected in a number of patient tissues. From a conceptual point of view, this might suggest that one of the nodules in low-grade myxofibrosarcoma becomes more dominant over time and turns into a dominant tumour nodule with a more histologically homogeneous growth pattern, though as other possibilities cannot be ruled out as only part of the tumour in selected cases was studied. Indeed, an attractive alternative hypothesis is a multifocal clonal evolution in myxofibrosarcoma and has already been shown in other neoplasms [48,49].

Lipid analysis of myxoid liposarcomas by imaging mass spectrometry revealed differences between low- and high-grade tumours (figure 3). The spectra from high-grade tumours revealed higher levels of phosphocholines. Phosphocholines are commonly detected by MALDI [48] and are thought to derive from (cell) membranes. The higher levels of these lipids in high grade tumours is consistent with the higher cellularity of higher grade tumours[50] (figure 3). These results are consistent with previous studies of Singer and coworkers which already demonstrated increased phosphocholines in high-grade myxoid liposarcoma using high resolution ex vivo NMR spectroscopy. These studies already demonstrated a correlation of lipid content and composition with the histology and grade in liposarcoma and how NMR-derived parameters can be used to classify liposarcoma subtypes [51,52]. These findings were underscored by our study using a different method (i.e. imaging mass spectrometry) in a different group of myxoid liposarcoma patient samples. Moreover, relative phosphocholine contents detected with high-resolution NMR spectroscopy predicted histological response of myxoid liposarcoma after treatment with troglitazone (a PPARγ agonist) [53]. Identification of lipids by imaging mass spectroscopy showed a predominant expression of PPARγ induced fatty acids in low-grade tumours such as triacylglycerols. PPARγ plays a key role in fatty acid synthesis[54]. Active PPARγ signalling promotes adipocytic differentiation in pre-adipocytes and is active in myxoid liposarcoma cells[24,55]. Our data suggest decreased PPARγ regulated fatty acid
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synthesis in myxoid liposarcoma upon increase in grade, histologically reflected in a diminished adipocytic phenotype, and consistent with recent work that established that PPARγ inactivation by the FUS/DDIT3 liposarcoma specific fusion protein is required for liposarcoma development[56].

Activation of PPARγ signalling also stimulated cell cycle withdrawal and PPARγ agonists have been shown to be promising in cancer treatment[57]. This is an attractive drug target as treatment options for myxoid liposarcoma patients with advanced disease are poor. However, phase II trials with rosiglitazone (a PPARγ agonist) in myxoid liposarcoma patients with advanced disease showed no effect on tumour size[58].

In conclusion, we have demonstrated that imaging mass spectrometry can distinguish between different types and different grades of myxoid tumours based solely on their biomolecular signatures, and can be used to identify proteins lipids specific to each type. Close examination of the data revealed previously unreported biomolecular changes consistent with the known tumour biology of myxofibrosarcoma and myxoid liposarcoma. Substantial intratumour heterogeneity was detected in the biomolecular profiles of myxofibrosarcoma tissues, consistent across multiple patient samples, that is consistent with the clonal selection model of myxofibrosarcoma tumour development. For myxoid liposarcoma tumour development is associated with inactivation of PPARγ by the FUS/DDIT3 fusion protein; PPARγ plays a crucial role in adipocytic differentiation and so led to the observed reduction in triacylglycerols with increasing tumour grade.

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Author contributions:

SW, AR and RZ carried out experiments. AR and LMD analyzed data. LMD, AD and PH designed experiments. SW, AR, LMD and PH wrote manuscript. All authors approved manuscript prior to submission.
Reference List

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