



Expression of multidrug resistance proteins, P-gp, MRP1 and LRP, in soft tissue sarcomas analysed according to their histological type and grade

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Abstract

The biological behaviour of different histological types and grades of soft tissue sarcomas (STS) varies. This might result in a differing sensitivity to cytotoxic drugs. Cross-resistance to functionally and structurally distinct natural-product drugs, known as multidrug resistance (MDR), is associated with the overexpression of P-glycoprotein (P-gp), multidrug resistance-associated protein 1 (MRP1) and lung resistance-related protein (LRP). The purpose of this study was to evaluate the expression of P-gp, MRP1 and LRP in STS according to their histological type and grade. In 141 chemotherapy-naïve STS patients, the expression of the three MDR proteins was detected by immunohistochemistry. Nine histological types were documented. These were 19% grade 1, 34% grade 2 and 47% grade 3 tumours. Expression of P-gp and LRP was observed more frequently than the expression of MRP1 ($P < 0.0001$). P-gp expression was most pronounced in malignant fibrous histiocytoma (MFH), but was low in leiomyosarcomas. MRP1 was expressed in most malignant peripheral nerve sheath tumours (MPNST). LRP was strongly expressed in MFH and unspecified sarcomas, but was low in liposarcomas. MRP1 and LRP expression was significantly more common in grades 2 and 3 compared with grade 1 tumours. P-gp expression was correlated with MRP1, especially in grade 3 STS. In conclusion, P-gp, MRP1 and LRP are expressed in the majority of STS, but this expression varies according to the histological type. MRP1 and LRP, but not P-gp expression, were found to be correlated to tumour grade. MDR might contribute to the observed differences in clinical behaviour within the heterogeneous group of STS.

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1. Introduction

Soft tissue sarcomas (STS) are a heterogeneous group of malignant tumours of mesenchymal origin. At least 19 distinct histological types and over 50 different subtypes have been recognised [1]. STS account for approximately 1% of all adult malignancies [2]. Because of their relatively low incidence, STS are often

considered as a single entity. However, large studies that have allowed subgroup analysis by histological type have revealed considerable differences in their biological behaviour [3–5]. In addition, recent clinical studies indicate that the response to chemotherapy is related to the histological (sub)type. For example, leiomyosarcomas have a poor response rate, while liposarcomas have a favourable response [3,4]. When the disease metastasises, the outcome after chemotherapy is poorer for patients with malignant fibrous histiocytomas (MFH) than for those with other histological types [4].

A relevant prognostic parameter for STS is histological grade. According to the system of the French Federation

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of Cancer Centers Sarcoma Group, histological grade corresponds to the degree of differentiation, mitotic rate and presence of tumour necrosis [6–8]. Patients with high grade tumours are at an increased risk of locally advanced disease and metastasis.

Doxorubicin and ifosfamide have the highest single agent activity in advanced STS, with response rates up to 20–30% [4,9–11]. However, a significant improvement in the survival rate has not been reported after doxorubicin or ifosfamide treatment, either as single agents or in combinations [12].

Multidrug resistance (MDR), whereby tumour cells are resistant to functionally and structurally unrelated natural-product drugs, may result in a failure to respond to chemotherapy treatment. MDR is associated with an increased expression of P-glycoprotein (P-gp) [13], multidrug resistance-associated protein 1 (MRP1) [14], and lung resistance-related protein (LRP) [15]. P-gp and MRP1 confer drug resistance by reducing the intracellular drug accumulation due to an active drug efflux. The spectrum of drugs expelled by MRP1 and P-gp is similar and includes anthracyclines, vinca-alkaloids and epipodophyllotoxins [16]. LRP is identified as the human major vault protein and may play a role in drug transport between the nucleus and cytoplasm [17]. Thereby, LRP alters the intracellular drug distribution, keeping the drug away from its target. The range of drugs associated with LRP is broader than that associated with P-gp and MRP1 and encompasses non-classical MDR substrates such as melphalan and platinum compounds [18,19].

Histological type and grade are thus linked to the outcome of patients. For this reason, it seems appropriate to analyse the MDR phenotype in STS separated by type and grade. The current study focuses on the expression of P-gp, MRP1 and LRP according to the histological type and grade of 141 chemotherapy-naïve STS patients.

2. Patients and methods

Criteria for inclusion in the current study were a chemotherapy-naïve primary tumour with a histological diagnosis of STS and the availability of paraffin-embedded tumour tissue. Cases were retrieved using the computerised files of the department of Pathology from the University Hospital, Groningen. The selected patients were diagnosed between 1979 and 1999.

The study group consisted of 141 STS, obtained from 70 male and 71 female patients (mean age: 48.7 years, median: 50 years, standard deviation: 18.8 years, range: 2–89 years). The histological diagnosis was made on haematoxylin-eosin stained paraffin sections, with or without additional immunohistochemical stains. Tumours were classified according to Enzinger and

Weiss [1]. There were 27 leiomyosarcomas (19%), 26 liposarcomas (18%), 18 MFH (13%), 14 rhabdomyosarcomas (10%), 12 synovial sarcomas (9%), 8 malignant peripheral nerve sheath tumours (MPNST)(6%), 6 fibrosarcomas (4%), 14 sarcomas not otherwise specified (NOS)(10%) and 16 other STS (11%). The distribution of histological types of the analysed group of tumours roughly reflects the general incidence of these STS [20].

STS were graded according to the grading system developed by Coindre and co-workers of the French Federation of Cancer Centers Sarcoma Group [5,7,8]. Additionally, grading was performed using the guidelines of the Association of Directors of Anatomic and Surgical Pathology [21]. These guidelines state that certain types (rhabdomyosarcomas, angiosarcomas) are high grade by definition. Although some have demonstrated that different grades between synovial sarcomas can be identified, this type was graded as 3 in our study. Types that were not graded in our study were epithelioid sarcomas ($n=3$), clear cell sarcomas ($n=2$) and alveolar soft part sarcoma ($n=1$).

2.1. Immunohistochemistry

From the available paraffin blocks, those containing the most viable parts of the tumour were selected. Immunohistochemistry was performed as previously described in Ref. [22]. After deparaffinisation, heat-induced epitope retrieval was performed. Samples were incubated with the primary antibody for one hour at room temperature. The following monoclonal antibodies were used: C494 (Signet Laboratories, Dedham MA, USA; dilution 1:200) to P-gp; MRPr1 to MRP1 (dilution 1:15) [23]; and LRP (Transduction Laboratories, Los Angeles CA, USA; dilution 1:400) to LRP. The staining procedure consisted of an indirect immunoperoxidase method using rabbit anti-mouse (C494, LRP) or rabbit anti-rat (MRPr1) peroxidase conjugated immunoglobulins (Dako, Glostrup, Denmark). Bound peroxidase was developed with diaminobenzidine and hydrogen peroxidase. Samples were counterstained with haematoxylin. Paraffin-embedded liver, lung and colon tissues served as positive controls for P-gp, MRP1 and LRP expression, respectively.

2.2. Scoring of immunoreactivity

The expression of P-gp, MRP1 and LRP was independently assessed by 4 observers, without knowledge of the clinical data. P-gp, MRP1 and LRP proteins were studied in adjacent slides. The distribution of P-gp, MRP1 and LRP expression was semi-quantitatively assessed by estimating the proportion of positively-stained tumour cells. According to previous studies, samples were considered negative for expression of each

of the proteins if less than or equal to 5% of the tumour cells were positive [24,25]. Positively scored samples were categorised using a 1-to-4 scale: 1+ for 6–25% positive tumour cells, 2+ for 26–50% positive tumour cells, 3+ for 51–75% positive tumours cells and 4+ for >75% positive tumour cells.

2.3. Statistics

The Wilcoxon signed ranks test was applied to compare the level of expression of the distinctive MDR proteins within the same specimens. The Mann–Whitney *U* test was used to analyse the differences in MDR expression between the histological types and grades. To quantify the correlation between MDR protein expression, the Spearman's rank test was used. A two-tailed *P*-value of <0.05 was considered to be significant. Statistical software, Statistical Package for the Social Sciences (SPSS) 10.0 for Windows (SPSS Incorporated, Chicago IL, USA) was used for the statistical analysis.

3. Results

3.1. MDR protein expression in the overall group of STS

Table 1 shows the distribution of scores for P-gp, MRP1 and LRP. One sample was not evaluable for P-gp and five samples were not evaluable for MRP1 because of a lack of representative tumour material.

P-gp expression was found in 110 of 140 analysed tumours (79%), MRP1 in 67/136 cases (49%) and LRP in 105/141 cases (74%). In the whole group of STS patients, expression of P-gp and LRP was significantly higher than MRP1 expression (both: $P < 0.0001$). In 136 of 141 tumours (96%), at least one of the three MDR proteins was detected.

Table 1
Scoring of expression for P-gp, MRP1 and LRP in the STS patients ($n = 141$)

	P-gp		MRP1		LRP	
Negative	30	21%	69	51%	36	26%
1	12	9%	14	10%	12	9%
2	28	20%	16	12%	25	18%
3	20	14%	14	10%	23	16%
4	50	36%	23	17%	45	32%
Total	140		136		141	
Missing	1		5		0	

Negative: $\leq 5\%$ positive tumour cells. 1+: 6–25% positive tumour cells. 2+: 26–50% positive tumour cells. 3+: 51–75% positive tumour cells. 4+: >75% positive tumour cells. (Percentages may not add up to 100% due to rounding of numbers.) STS, soft tissue sarcomas. P-gp, P-glycoprotein. MRP1, multidrug resistance-associated protein 1. LRP, lung resistance-related protein.

For 135 tumours, immunohistochemical results were available for all three MDR proteins. In these 135, expression of all three proteins was found in 41 cases (30%), whereas five tumours (4%) scored negative for P-gp, MRP1 and LRP (two leiomyosarcomas, two myxoid liposarcomas and one synovial sarcoma). Co-expression of P-gp and MRP1 was found in 58/135 STS (43%), co-expression of P-gp and LRP in 79/140 cases (56%), and co-expression of MRP1 and LRP in 51 of the 136 analysed STS (38%).

When the semi-quantitative scores (negative, or positive 1+ to 4+ were analysed), P-gp expression correlated with MRP1 expression (Spearman's correlation coefficient 0.35; $P < 0.0001$). No significant correlation existed between P-gp and LRP, or between MRP1 and LRP.

3.2. MDR protein expression in the major histological types of STS

Figs. 1, 2 and 3 show the expression of P-gp, MRP1 and LRP in the different histological types, respectively. Only groups consisting of more than 10 cases are discussed hereafter.

3.2.1. Leiomyosarcomas ($n = 27$)

Leiomyosarcomas represented the largest group in the current study. All leiomyosarcomas were located at an extremity; no gastrointestinal leiomyosarcomas were included. Seventeen of 27 samples were P-gp negative (63%), significantly more than any other histological type ($P < 0.001$). Most leiomyosarcomas were negative for MRP1: 16/25 samples (64%). Two samples were not evaluable for MRP1. LRP was widely expressed in the leiomyosarcomas: 24 of 27 samples were positive (89%). Most samples had abundant LRP expression (3+ or 4+ in 63% of the cases)

3.2.2. Liposarcomas ($n = 26$)

Most liposarcomas were positive for P-gp (22/26; 85%), with 27% of the cases in each of the categories of 2+, 3+ and 4+. MRP1 immunoreactivity was present in 10/26 samples (38%), of which five were in the lowest category (1+). Like MRP1, LRP expression was absent in most liposarcomas: 15/26 scored negative (58%).

In addition, liposarcomas were analysed for subtype (well-differentiated, myxoid, round cell and dedifferentiated), as this is associated with biological and clinical behaviour. The distribution of the subtypes was: six well-differentiated, 11 myxoid, two round cell and seven dedifferentiated liposarcomas. It was noted that the number of LRP-negative samples in the overall group of liposarcomas was mainly due to the myxoid subtype, with 10 of 11 samples being negative. P-gp and MRP1 expression did not differ significantly between the subtypes.

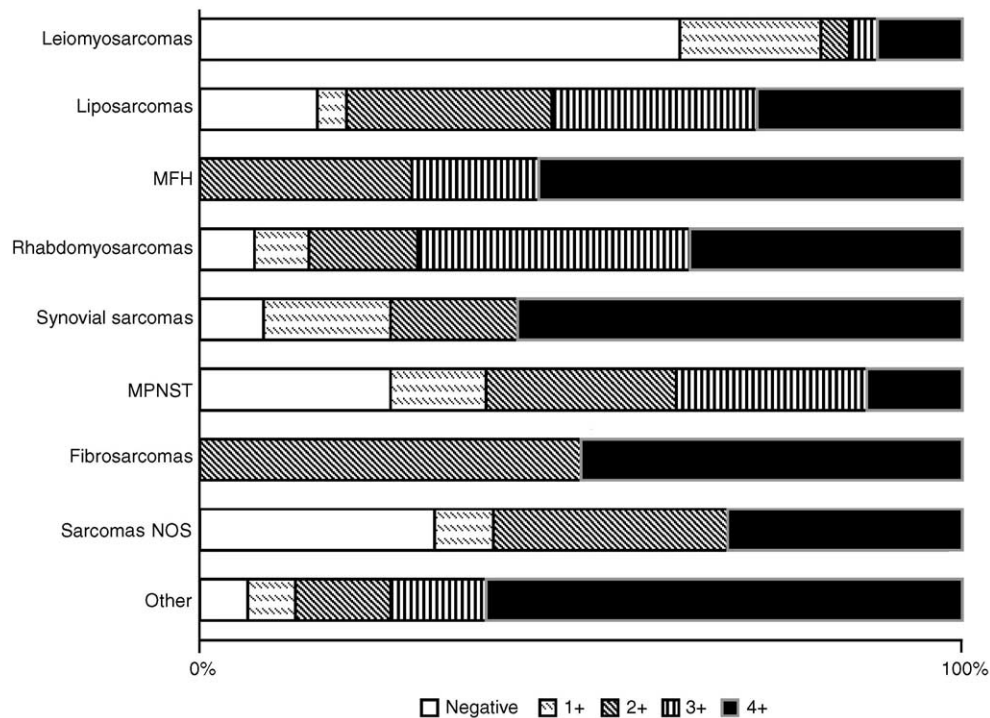


Fig. 1. Expression of P-gp per histological type. MFH, malignant fibrous histiocytes; MPNST, malignant peripheral nerve sheath tumours; NOS, not otherwise specified.

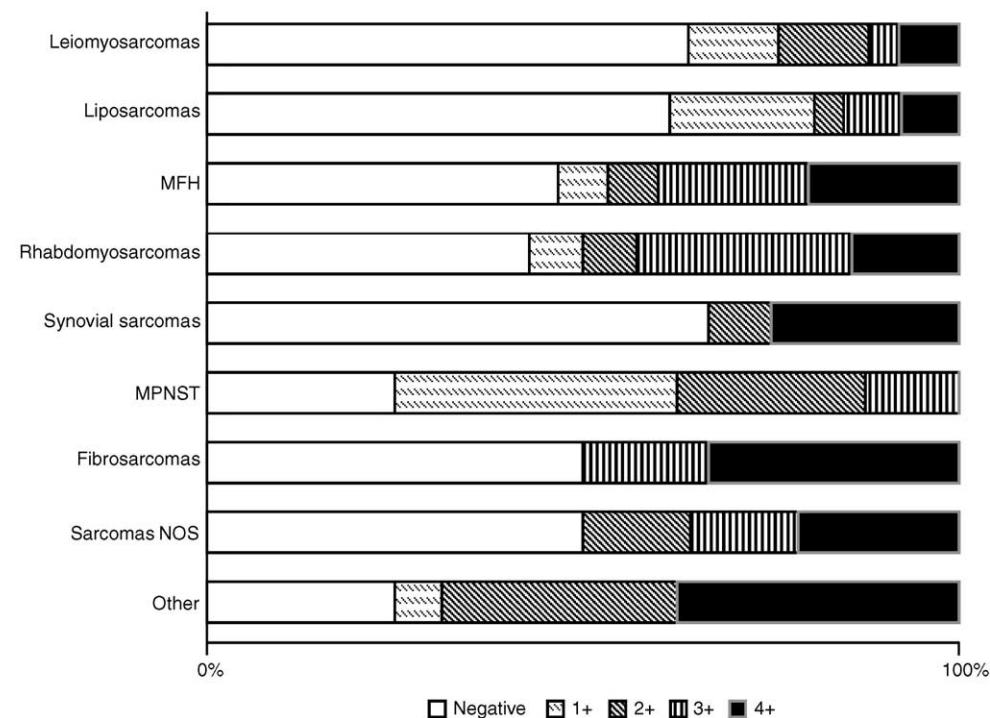


Fig. 2. Expression of MRP1 per histological type.

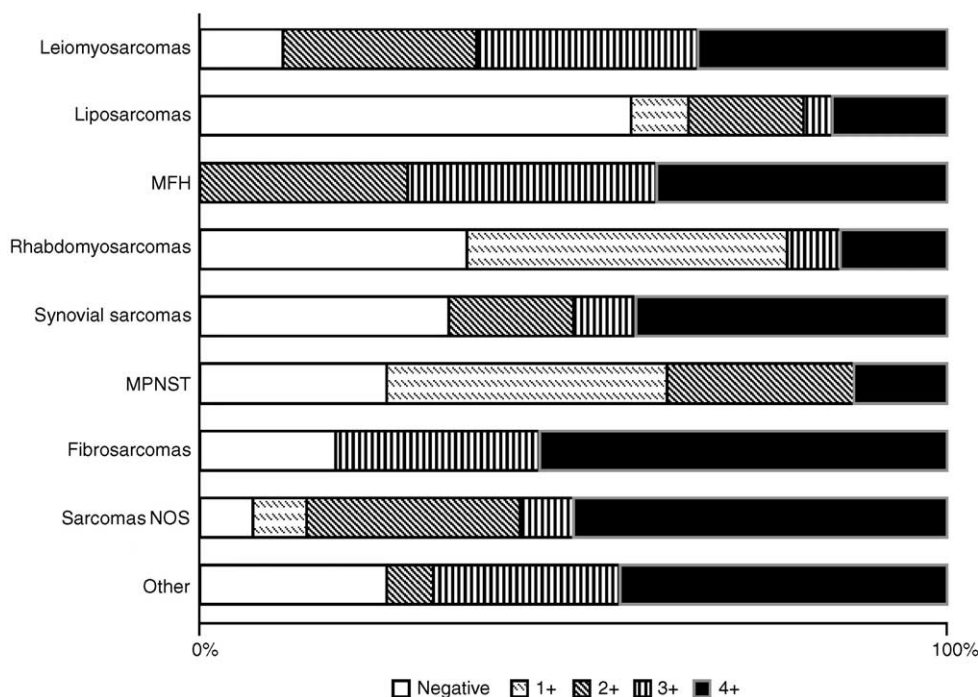


Fig. 3. Expression of LRP per histological type.

3.2.3. MFH (n = 18)

Most MFH were found to have extensive P-gp expression (72% 3+ or 4+). No samples were negative for P-gp. MRP1 staining was less widespread with 47% of the samples staining negative. Three samples could not be evaluated for MRP1 expression. Like P-gp, LRP expression was abundant: there were no negative samples and 72% of the cases were 3+ or 4+.

3.2.4. Rhabdomyosarcomas (n = 14)

Widespread P-gp staining was common in the rhabdomyosarcomas: 71% of the tumours were 3+ or 4+. Only one sample completely lacked immunoreactivity towards P-gp. MRP1-negative and -positive samples were evenly distributed: 43% versus 57%. Most positive samples had over half of their tumour cells showing immunoreactivity to the MRP1 antibody. LRP was absent or low in all, but three, samples.

Like with the liposarcomas, the subtype of rhabdomyosarcoma is associated with varying biological behaviours. In this limited series, no difference in P-gp and MRP1 expression could be observed for the separate subtypes of rhabdomyosarcomas (embryonal, alveolar and pleomorphic). The two samples with >75% P-gp positive tumour cells (4+) were both pleomorphic rhabdomyosarcomas.

3.2.5. Synovial sarcomas (n = 12)

Fifty-eight percent of the synovial sarcomas had virtually all of their tumour cells showing immunoreactivity to P-gp (4+). In contrast, over 67% of the

samples was completely negative for MRP1. The categories of LRP expression were evenly distributed in this group of synovial sarcomas.

3.2.6. Unspecified type (sarcoma NOS; n = 14)

Amongst sarcomas NOS, a heterogeneous pattern for P-gp expression was observed; one sample was not evaluable for P-gp staining. Fifty percent of the samples were negative for MRP1, the remainder being fairly equally distributed in the 2+, 3+ and 4+ groups. Half of the tumours had the maximal score for LRP staining; only one sample was LRP-negative.

3.2.7. Other types (n = 16)

The heterogeneous group of rare histological types was lumped together for descriptive reasons. Subtypes included were: angiosarcomas (n = 4), epithelioid sarcomas (n = 3), clear cell sarcomas (n = 2), gastrointestinal stromal tumours (GIST) (n = 2), PPNET/Ewing's sarcoma (n = 2), malignant haemangiopericytoma (n = 1), myxoid chondrosarcoma (n = 1), alveolar soft part sarcoma (n = 1). This group was characterised by a high P-gp expression (75% 3+ and 4+). No clear pattern was observed for MRP1 and LRP expression.

3.3. Histopathological grading of the STS

Of the 141 tumours, 135 were graded according to the aforementioned guidelines. This resulted in 25 grade 1 (19%), 46 grade 2 (34%) and 64 grade 3 STS (47%).

Amongst the grade 1 tumours were 15 liposarcomas (six well-differentiated and nine myxoid liposarcomas), eight leiomyosarcomas, one fibrosarcoma and one malignant haemangiopericytoma.

Grade 2 tumours were: leiomyosarcomas ($n=14$), MFH ($n=9$); liposarcomas ($n=8$), MPNST ($n=6$), sarcoma NOS ($n=5$), fibrosarcomas ($n=2$) and other types ($n=2$).

Grade 3 tumours were: rhabdomyosarcomas ($n=14$), synovial sarcomas ($n=12$), MFH ($n=9$), sarcoma NOS ($n=9$), leiomyosarcomas ($n=5$), fibrosarcomas ($n=3$), liposarcomas ($n=3$), MPNST ($n=2$) and other types ($n=7$).

3.4. MDR protein expression in relation to histopathological grade

Expression of the evaluated MDR proteins in relation to histopathological grade is shown in Table 2. P-gp expression was equally distributed over the different grades; however, MRP1 and LRP expression was significantly higher in grades 2 and 3 tumours when compared with grade 1 tumours ($P=0.007$ for MRP1; $P=0.003$ for LRP). The correlation between P-gp and MRP1, as observed for the overall group, was more pronounced in the grade 3 tumours with a Spearson's correlation coefficient of 0.54 ($P<0.0001$).

Co-expression of P-gp and MRP1 ($P=0.011$), P-gp and LRP ($P<0.0001$) was significantly more frequently observed in grades 2 and 3 STS compared with grade 1. Co-expression of MRP1 and LRP was not statistically different between grades 2 and 3 STS versus grade 1.

4. Discussion

Despite the many different histological types of STS, traditionally they have been lumped together as if they are a single entity. However, marked differences in biological behaviour have underscored the importance of distinguishing the different histological types. Although the response to chemotherapy is rather limited in STS,

responses are not strictly uniform, suggesting different mechanisms of resistance in different histological types. The present study evaluated the expression of P-gp, MRP1 and LRP in 141 chemotherapy-naïve primary STS patients.

P-gp expression might be associated with a poor response to chemotherapy in childhood rhabdomyosarcomas as well as in adult STS, although the results in different studies are conflicting [25–29]. MRP1 has been detected in STS and co-expression of MRP1 and P-gp expression has been demonstrated to be associated with tumour grade [30]. The combined expression of P-gp, MRP1 and LRP in the various histological types of STS has not been described before.

P-gp expression was found in the vast majority of STS analysed in this study (79%). Previous immunohistochemical studies have reported marked differences in the percentage of P-gp-positive STS, ranging from 0 to 100% [22,27–29,31,32]. These differences can be attributed to methodological variations in the immunohistochemical techniques used (tissue preparation and storage, epitope retrieval, antibodies used), inclusion of different histological types and limited numbers of samples per group.

Remarkably few leiomyosarcomas expressed P-gp when compared with the other histological types. This appears to contradict earlier reports [27,30]. However, previous studies may well have been confounded by the inclusion of GISTs, as these were previously assumed to be leiomyosarcomas. It has now become evident that GISTs are a biologically and clinically distinct type [33]. GISTs are very chemoresistant, in contrast to true leiomyosarcomas [34].

A high expression of P-gp in MFH, liposarcomas and synovial sarcomas, observed in previous studies, was confirmed in our study. The criticism with regard to the diagnosis “MFH” (some consider MFH a waste basket of several different types) is brought into perspective by a recent study that designated MFH as a distinctive group of STS [35]. This implies that evaluating MFH is still meaningful. MFH have a poor response rate to doxorubicin-based therapy [4], while the current study

Table 2
P-gp, MRP1 and LRP expression in STS patients according to their histological grade

	P-gp						MRP1						LRP					
	Grade 1		Grade 2*		Grade 3		Grade 1		Grade 2*		Grade 3*		Grade 1		Grade 2		Grade 3	
Negative	9	36%	12	27%	8	13%	18	72%	22	50%	27	44%	12	48%	5	11%	18	28%
1+	2	8%	3	7%	7	11%	4	16%	5	11%	4	7%	2	8%	3	7%	7	11%
2+	5	20%	9	20%	13	20%	1	4%	7	16%	6	10%	4	16%	11	24%	9	14%
3+	2	8%	9	20%	9	14%	1	4%	4	9%	9	15%	5	20%	8	17%	10	16%
4+	7	28%	12	27%	27	42%	1	4%	6	14%	15	25%	2	8%	19	41%	20	31%

Grading performed according to Trojani, Coindre and coworkers of the French Federation of Cancer Centers Sarcoma Group and using the guidelines of the Association of Directors of Anatomic and Surgical Pathology. Negative: $\leq 5\%$ positive tumour cells, 1+: 6–25%, 2+: 26–50%, 3+: 51–75%, 4+: $> 75\%$. (Percentages may not add up to 100% due to rounding of numbers.)

* Data is missing for some samples.

revealed a substantial expression of MDR proteins, in particular P-gp and LRP. Liposarcomas, that tend to respond better to doxorubicin-based treatment, differed from MFH especially in their expression of LRP.

Although a correlation between P-gp expression and tumour grade was observed in some studies, this could not be confirmed in the present study [27,30]. Nakanishi and colleagues reported a relationship between tumour grade and P-gp expression when comparing high-grade tumours to low and intermediate grade STS [27]. However, a relatively high proportion of MFH (24/55, 44%) were included in their study, which are usually of intermediate or high grade. Therefore, their findings appear to be in concordance with the present study in which all MFH were P-gp positive and had the most extensive P-gp expression. This illustrates again that the relationship between tumour grade and P-gp expression might well depend on the histological types included.

Limited data are available on MRP1 expression in STS. In the present group, MRP1 expression was detected in only 49% of cases and co-expression with P-gp was observed in only 43%. Oda and colleagues found co-expression of MRP1 and P-gp mRNA in 38% [30]. In their study, a correlation of tumour grade with the co-expression of P-gp and MRP1 mRNA was found. In the present study, P-gp and MRP1 co-expression was observed significantly more often in the intermediate and high-grade compared with the low-grade STS.

For the overall group, expression of P-gp and MRP1 was moderately correlated, but this correlation became more pronounced when only grade 3 tumours were considered. Furthermore, the present study showed that in the overall group of STS, MRP1 expression was significantly less frequent than P-gp and LRP expression.

Data are scarce on LRP expression in STS. In a comparative study of GIST and leiomyosarcomas, Plaat and colleagues found significantly higher LRP expression in the GIST group [33]. In a study of locally advanced STS, LRP positivity was found in 64% of the cases [22]. Kusakabe and colleagues reported two epithelioid sarcomas were positive for LRP, but not for P-gp or MRP [36]. Cell lines derived from these epithelioid sarcomas exhibited an MDR phenotype in the absence of P-gp and MRP1, suggesting a role for LRP. One in vitro study has demonstrated the presence of LRP in 5 of 7 rhabdomyosarcoma cell lines [37]. In the current study, 74% of the STS were LRP-positive. Expression of LRP was not correlated to P-gp or MRP1 expression. This suggests that the expression of LRP is regulated by factors other than the membrane efflux pumps P-gp and MRP1. Focusing on liposarcomas, all but one of the myxoid subtype were negative for LRP. Myxoid liposarcomas have been previously described to have low or no expression of LRP, possibly due to chromosome breakage near the *LRP* gene [38]. Liposarcomas other than the myxoid subtype often expressed LRP. The implications of these findings remain speculative, because well-differentiated liposarcomas rarely metastasise and have therefore probably not been included in the study evaluating chemotherapy, whereas other non-myxoid liposarcomas are rare [1]. As the well-differentiated liposarcomas are surgically treated, it is conceivable that MDR has no clear clinical implications for this particular type.

Grades 2 and 3 tumours expressed significantly more MRP1 and LRP than grade 1 tumours. In the recent version of the American Joint Committee on Cancer (AJCC) staging system, grades 2 and 3 are considered together and contribute to patients' prognosis unfavourably [39]. Moreover, co-expression of P-gp and MRP1 and of P-gp and LRP was significantly more often observed in grades 2 and 3 patients. Despite the initial tumour shrinkage observed, especially in high-grade STS, the poor overall survival for patients with high-grade tumours suggests that MDR is a clinically relevant problem. With the observed correlations, MRP1 and LRP expression seem to be indicative of a higher degree of malignancy.

In conclusion, the expression of P-gp, MRP1 and LRP varies between different histological types and grades of STS and it is conceivable that this might contribute to the differences observed in the response to chemotherapy and the outcome of patients with STS.

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