Alma Mater Studiorum – Università di Bologna

DOTTORATO DI RICERCA IN

ONCOLOGIA, EMATOLOGIA E PATOLOGIA

Ciclo XXXIV

Settore Concorsuale: 06/D3

Settore Scientifico Disciplinare: MED06

Microenvironment and prognostic factors in bone tumors: IDH mutations in chondrosarcoma

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Esame finale anno 2022

To Dr Gaetano Bacci,
who instilled in me the passion
for research and cure of bone cancers.

To Sara and Elia,
who enjoy the beauty in discovering.

ABSTRACT

Microenvironment in bone tumors is a dynamic entity composed of cells from different origins (immune cells, stromal cells, mesenchymal stem cells, endothelial cells, pericytes) and vascular structures surrounded by a matrix of different nature (bone, cartilage, myxoid).

Interactions between cancer cells and tumor microenvironment (TME) are complex and can change as tumor progress, but are also crucial in determining response to cancer therapies.

Chondrosarcoma is the second most frequent bone cancer in adult age, but its treatment still represents a challenge, for the intrinsic resistance to conventional chemotherapy and radiation therapy. This resistance is mainly due to pathological features, as dense matrix, scarce mitoses and poor vascularization, sustained by biological mechanisms only partially delucidated.

Somatic mutation in the Krebs cycle enzyme 3andomized dehydrogenase (IDH) have been described in gliomas, acute myeloid leukemia, cholangiocarcinoma, melanoma, colorectal, prostate cancer, thyroid carcinoma and other cancers. In mesenchymal tumors IDH mutations are present in about 50% of central chondrosarcoma. IDH mutations are an early event in chondrosarcoma-genesis, and contribute to the acquisition of malignancy through the block of cellular differentiation, hypoxia induction through HIF stabilization, DNA methylation and alteration of cellular red-ox balance.

While in gliomas IDH mutations confers a good prognosis, in chondrosarcoma IDH prognostic role is controversial in different reported series.

First aim of this project is to define the prevalence and the prognostic role of IDH mutation in high grade central conventional chondrosarcoma patients treated at Istituto Ortopedico Rizzoli.

Second aim is the critical revision of scientific literature to understand better how a genomic event in cancer cell can trigger alteration in the TME, through immune infiltrate reshaping, angiogenesis induction, metabolic and methylation rewiring.

Third aim is to screen other sarcoma histotypes for the presence of IDH mutation.

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6. Introduction

6.3 Chondrosarcoma

Cartilaginous tumors, as described in the World Health Organization (WHO) classification [1] are a group of benign and malignant lesions with cartilaginous differentiation, and in the whole represent the most common primary neoplasms affecting bone. [TABLE 1]

The most common entity is conventional chondrosarcoma, accounting for 85%, which is sub-classified in central conventional chondrosarcoma, if arises in the medulla of the bone, and peripheral conventional chondrosarcoma, if it starts in the periphery of the bone. This two entities can arise as the transformation of benign bone lesions (enchondroma and osteochondroma respectively) through different molecular mechanisms (IDH1/2 for central, EXT1/2 for peripheral), and in this case are defined as "secondary" chondrosarcoma [2].

Dedifferentiated chondrosarcoma, can be considered as the end of the spectrum of the malignant transformation of the conventional type, and characteristically is composed by a well-differentiated cartilaginous tumor (which can be enchondroma or grade 1 or 2 chondrosarcoma), sharply demarked from a high grade malignancy which presents dedifferentiated features resembling osteosarcoma or undifferentiated pleomorphic sarcoma, or, less frequently, a high-grade angiosarcoma, leiomyosarcoma or rhabdomyosarcoma [3].

Other histologic types are periosteal chondrosarcoma, clear cell chondrosarcoma and mesenchymal chondrosarcoma.

Table 1-WHO classification of Tumors 5th edition: Chondrogenic tumors

Benign	Intermediate	Malignant
Subungueal exostosis	Chondromatosis NOS	Central chondrosarcoma G 2-3
Bizarre paraosteal osteochondromatous proliferation	Atypical cartilaginous tumor/Central chondrosarcoma G 1	Secondary peripheral chondrosarcoma G2-3
Periosteal chondroma	Atypical cartilaginous tumor/Secondary peripheral chondrosarcoma G1	Periosteal chondrosarcoma
Enchondroma		Clear cell chondrosarcoma
Osteochondroma		Mesenchymal chondrosarcoma
Chondroblastoma NOS		Dedifferentiated Chondrosarcoma
Condromyxoid fibroma		
Osteochondromyxoma		

The incidence of central high grade chondrosarcoma is estimated around 1.8 cases per 1 million person-years. It affects adults in the third to six decades of life, but when associated to enchondromatosis (Ollier and Maffucci Syndromes, non-hereditary conditions in which enchondromatosis is combined with vascular tumours) it occurs in in younger age. Patients with these conditions have a 40-50% risk to develop chondrosarcoma [4].

Radiographic appearance is usually of large osteolytic areas with permeative bone destruction, calcifications, and soft tissue extension, which can be confirmed on CT scan. MRI is the method of choice to identify typical features as abundant mucoid areas. PET-CT could be helpful to recognize the tumor grade and if distant metastases are present.

Histopatology shows a lobular configuration with pre-existing bone trabeculae entrapping. Tumor cells are embedded within the cartilaginous matrix which can be hyaline or, more often, present myxoid changes. Nuclei can be small and condensed or present open chromatin and a visible nucleolus, mitoses can be detected. At the periphery of less differentiated lobules cells are spindled and gradually merge with fibrous cells of the septations surrounding the lobules, which contain numerous small vascular channels and immune cells. At immunohistochemistry S100 positivity can be found, with brachyury negativity, IDH mutation can be detected with specific antibodies only in a limited amount of IDH mutated tumors. Molecular detection of IDH mutation may be helpful in the differential diagnosis with chondroblastic osteosarcoma.

Tumor grade is defined on the basis of cellularity, degree of nuclear atypia, hyperchromasia, nuclear size, myxoid matrix changes, and number of mitoses. [Fig. 2]

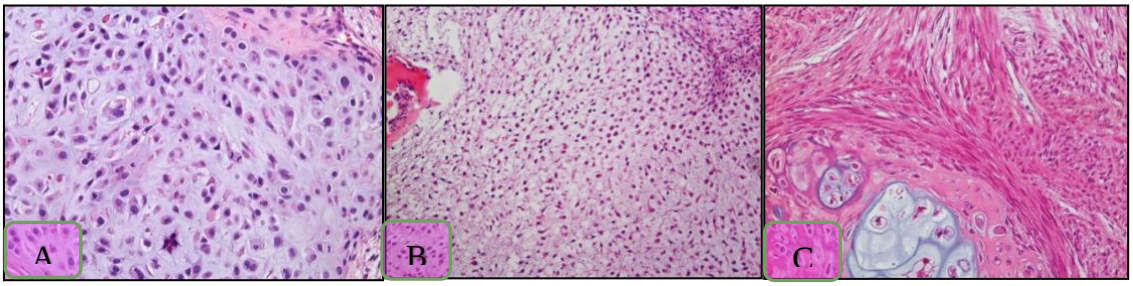


Figure 2: Central conventional chondrosarcoma grade 1 (A), grade 2 (B), grade 3 (C).

Tumor grade and stage are the main prognostic factors.

The reported 5 years overall survival (OS) rate is 74-99% for grade 2, and 31-77% for grade 3.

The 10 years OS rate is 58-86% for grade 2 and 26-55% for grade 3.

Local recurrence rate are 19% for grade 2 and 26% for grade 3. Metastases occurs in 10-30% of grade 2, and in 32-71% of grade 3.

Surgery is the principal therapy, while chemotherapy and radiotherapy have a limited role, since chondrosarcomas are resistant to these conventional therapy [5,6]. The relatively poor vascularity, low pH and increased interstitial pressure, are potential obstacles to drug delivery [7]. The understanding of molecular mechanisms involved in the progression to chondrosarcoma is crucial for the discovering of new therapeutic approaches.

1.2 Pathogenesis of conventional chondrosarcoma

The event causing the transformation from normal tissue to pathologic lesion it's believed to happen during the process termed endochondral ossification, in which blood vessels invade the cartilage matrix and bring osteoblasts for bone production at the center of the cartilaginous template. Most of the cartilage template is replaced by bone except the growth plate, where chondrocytes continue to undergo a coordinated process of differentiation (from the resting phase, through proliferating, prehypertrophic and hypertrophic stages, to programmed cell death) providing the scaffolding on which new bone is formed [8].

Benign cartilage tumors develop during periods of bone growth in the metaphyseal region, adjacent to the growth plate, suggesting that they arise from the failure of terminal differentiation of growth plate chondrocytes. Parathyroid hormone-like hormone (PTHLH), produced by perichondrial cells, Indian Hedgehog (IHH), and the transcription factor GLI3 are involved in the differentiation process. [9-12]. 8% of patients with enchondromatosis harbor a mutation in the gene encoding the PTHLH receptor, PTHR1, in their tumor tissue.

Osteochondromas have a cartilage cap, composed of a mixture of normal and mutated cells, that grows while the growth plate is active, and cease to grow when it closes at puberty. In multiple osteochondromas syndrome (MO) [14] malignant transformation occurs in 1-5% of cases. MO is inherited in an autosomal dominant manner, and linkage studies have identified causative genes. Inactivating mutations are found in either the gene encoding exostosin 1 (EXT1) at 8q24 (65%) or EXT2 at 11p11-13 (35%) [15]. EXT1 and EXT2 are involved in heparan sulphate biosynthesis [16], mutations in that genes cause accumulation of heparan sulphate

proteoglycans in the cytoplasm, instead of in the extracellular space, resulting in an abnormal diffusion of hedgehog ligands in the extracellular environment, resulting in a larger area in which IHH can diffuse. This change in diffusion area could potentially result in the loss of polar organization allowing growth-plate chondrocytes to grow in the wrong direction.

Despite their histological similarities, different pathogenic processes between central and peripheral chondrosarcomas suggest that therapeutic targets might differ between these two subtypes.

The activation of the hedgehog pathway is an early event in tumor formation and progression and raises the possibility that therapeutic targeting of hedgehog signaling pathways could be used to induce cells into a more differentiated, less aggressive state.

Mechanisms that lead to progression from enchondroma to chondrosarcoma may represent good options for therapeutic targeting. In particular, alteration of p53 or Rb pathways are associated with increasing histological grade and described in 95% of high grade chondrosarcoma. Related to these pathways are other potential targets [8]:

- Insulin-like growth factor (IGF) pathway, caused by the loss of p53
- Amplification of 12q13 region, which harbors MDM2
- Loss of 9p21 region, which includes CDKN2A locus which encodes for InK4A and ARF (linked to p53 and Rb pathways)
- CDK4, involved in the Rb pathway
- mTOR signaling
- P13K-AKT and sRC pathways
- HIF1α expression, induced by P13K and sRC
- COL2A1, encoding the alpha chain of type 2 collagen fibers
- YEATS2, EGFR, NRAS
- Genes associated with glycolysis, and galectin 1
- BCL2
- Matrix metalloproteinases (MMPs)
- Expression of multidrug resistance genes such as P-glycoprotein

1.3 IDH mutations in cancer

Mutation in the isocitrate dehydrogenase (IDH) gene has been first identified in colon cancer in 2006 [17], and then described in a whole-exome sequencing study of 22 glioblastomas [18]. Subsequent studies revealed that mutations in IDH1 or IDH2 are prevalent in various types of cancer, including low-grade glioma and secondary glioblastoma (80%) [19,20], acute myeloid leukaemia (AML; 20%)[21-23], cholangiocarcinoma (20%)[24-25], chondrosarcoma (80%)[26], sinonasal undifferentiated carcinoma (49–82%)[27-29] and angioimmunoblastic T cell lymphoma (32%)[30-31], suggesting a pathogenetic role for such mutations.

IDH mutations are single amino acid substitutions at the arginine 132 residue (R132) in IDH1, and at the analogous residue arginine 172 (R172) of IDH2 or arginine 140 (R140) in IDH2.

As hotspot mutations that occur early in tumorigenesis with uniform and specific expression in tumor cells [32,33], IDH mutations constitute appealing therapeutic targets. To this end, small-molecule inhibitors of mutant IDH, mutant IDH-directed immunotherapies, and agents targeting mutant IDH-induced metabolic liabilities are active areas of research and the focus of clinical trials in patients with IDH-mutant cancers [TABLE 2].

Three isoforms of IDH are described: IDH1 is localized in the cytosol and IDH2 and 3 in the mitochondria. IDH catalyzes the reversible oxidative decarboxylation of isocitrate to α-ketoglutarate (α-KG) while reducing NADP+ to NADPH. When heterozygous mutations occur, the enzyme gains a neomorphic activity leading to conversion of α-KG into the oncometabolite D-2-hydroxyglutarate (D-2HG) in a process that consumes rather than produces NADPH and instead generates NADP+ [34,35]. This leads to the alteration of the red-ox cellular balance.

The maintenance of the heterozygous state is essential for D-2HG production [36-38]. The wild-type IDH proteins form homodimers that can transition between an inactive open state, an inactive semi-open state ,and a catalytically active closed conformation. The presence of a mutant IDH subunit in the enzymatic complex favors the closed conformation and confers a high affinity for NADPH, with subsequent reduction of α-KG to D-2HG [36,40]. For D-2HG production a balanced ratio of wild-type and mutant alleles is required [39, 40-41].

The biological effects of IDH mutations are due to the structural similarities between D-2HG and α-KG, which lead, through the competitive inhibition of cytoplasmic α-KG-dependent dioxygenases [42-44], to epigenetic dysregulation with aberrant histone and DNA methylation,

chromatin restructuring, blocking of cellular differentiation, HIF1α stabilization, and angiogenesis. [45-47].

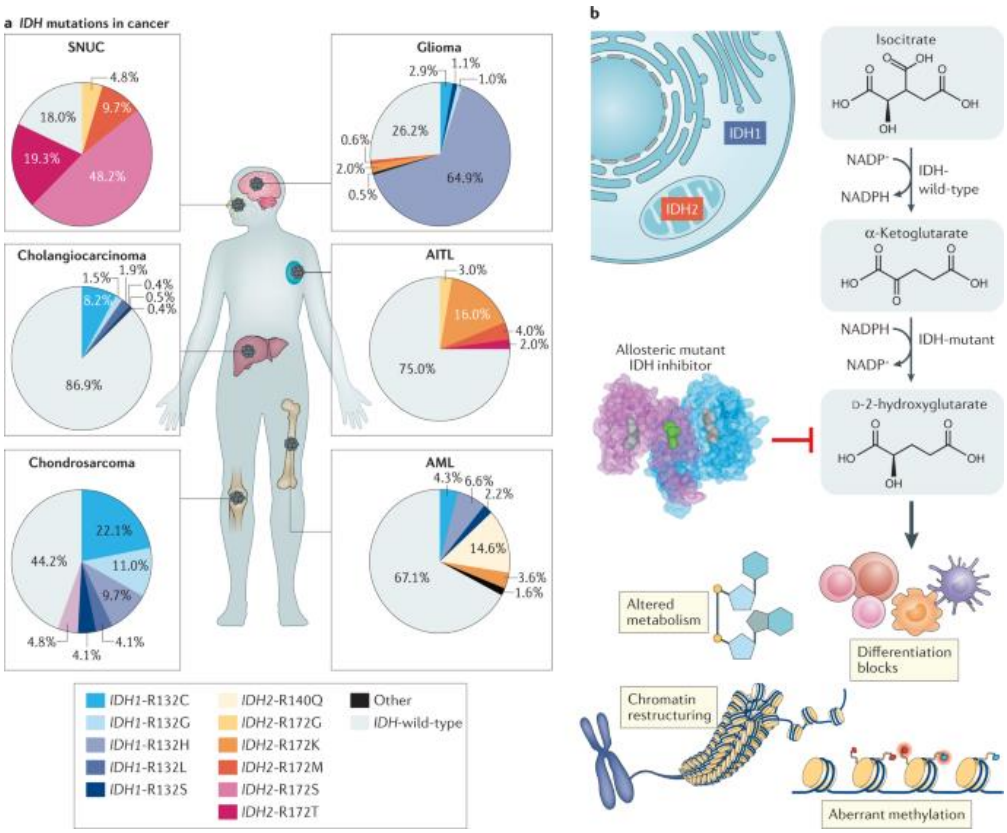


Figure 2: clinical and biological role of IDH mutations in cancer (from Pirozzi CJ, Hai Yan et al, Nature 2021) [66]

The clinical implication of IDH mutations differs among the different cancer types.

IDH1 is the most frequently mutated gene in low-grade gliomas, with the R132H mutation being the most common [48]. The presence of IDH mutation confers a better prognosis to patients with glioma: the median OS duration has been reported to be 51 months versus 22 months among patients with grade III astrocytomas and 31 months versus 13 months among those with grade IV glioblastomas [49].

In AML, IDH1 mutations (R132C and R132H) have been found in 20% of the patients, while IDH2 mutations (R172 and R140) are more frequent. The prognostic role of IDH mutations in AML has been investigated in a meta-analysis of data from 33 studies involving 12,747 patients. Interestingly, the subgroup of patients with IDH1-mutant AML had worse OS and EFS, and a reduced CR rate than those with IDH-wild-type disease; while patients with IDH2 mutant AML had better OS, although, those with IDH2-R172 mutations had reduced CR rates. Other studies

have found no prognostic implications for IDH mutations in AML [50].

In cholangiocarcinoma IDH1 mutations (R132 and rarely R172) occur more frequently in intrahepatic bile ductular or small duct cholangiocarcinoma [51,52]. Several studies assessing the prognostic implications of IDH mutations in cholangiocarcinoma have revealed conflicting survival outcomes: one study reports longer OS and reduced rate of recurrence at 1 year for IDH1mut versus IDHwt cholangiocarcinoma (10.5% vs 41.7%) whereas other studies found no statistically significant differences in survival according to IDH mutational status [25].

TABLE 2 | **Results of trials of IDH-targeted therapies for *IDH*-mutant cancers reported to date (from Pirozzi CJ, Nature Review 2021) [66]**

Population	Treatment	Study phase	Efficacy results	Most common grade ≥3 TraEs	Ref.
27 patients with *IDH1*-mutant AML	BAY-1436032	I	**ORR 14.8%; median OS 6.6 months**	Fatigue (3.7%); differentiation syndrome (3.7%)	53
258 patients with *IDH1*-mutant haematological malignancies, including 242 with AML, of whom 179 had R/R AML	Ivosidenib	I	**ORR 41.6%; CR rate 21.6%; median OS 8.8 months**[a]	QT prolongation (7.8%); differentiation syndrome (3.9%); anaemia (2.2%); thrombocytopenia (3.4%); leukocytosis (1.7%)[b]	54
23 patients with newly diagnosed *IDH1*-mutant AML ineligible for intensive induction chemotherapy	Ivosidenib + azacitidine	Ib/II	**ORR 78.3%; CR rate 60.9%;** estimated 12-month OS 82.0%	Neutropenia (21.7%); anaemia (13.0%); QT prolongation (13.0%); leukocytosis (8.7%); differentiation syndrome (8.7%)	55
17 patients with *IDH1*-mutant AML and 2 patients with *IDH1*-mutant high-risk MDS	Ivosidenib and venetoclax ± azacitidine	Ib/II	**ORR 88.9%; CR rate 38.9%**[c] Among 9 patients with R/R AML: median OS 9.7 months	Differentiation syndrome (5.3%); tumour lysis syndrome (5.3%)	56
Patients with newly diagnosed *IDH1*-mutant AML (*n* = 60) or *IDH2*-mutant AML (*n*=91)	Ivosidenib or enasidenib with induction and consolidation therapy	I	**CR rate 55.0%** (ivosidenib) **and 47.3%** (enasidenib)	TRAEs not defined; however, grade ≥3 differentiation syndrome occurred in 2.0% of patients overall; grade ≥3 QT prolongation occurred in 2.9–10.0%	57
345 patients with *IDH2*-mutant haematological malignancies, predominantly R/R AML (*n* = 280) or high-risk MDS(*n*=17)	Enasidenib	I/II	**ORR 38.8%; CR rate 19.6%;** median EFS 4.7 months; median OS 8.8 months[d]	Hyperbilirubinaemia (10.0%); thrombocytopenia (7.0%); differentiation syndrome (6.0%)	58
21 patients with *IDH1*-mutant R/R AML and 3 patients with *IDH1*-mutant high-risk MDS	IDH305	I	In patients with AML: ORR 33.3%; CR rate 9.5%	Increased serum bilirubin (4.2%)	59
35 patients with *IDH1*-mutant R/R AML or MDS	Olutasidenib ± azacitidine	I/II	Olutasidenib monotherapy (*n* = 16): CR rate 12.5%. Combination therapy (*n* = 11): CR rate 18.2%	Across monotherapy and combination groups: febrile neutropenia (22.9%); anaemia (20.0%); pneumonia (17.1%); differentiation syndrome (14.3%)	60

73 patients with previously treated advanced-stage *IDH1*-mutant cholangiocarcinoma	Ivosidenib	I	**ORR 5.5%; SD rate 56.2%;** median PFS 3.8 months; 6-month PFS 40.1%; 12-month PFS 21.8%; median OS 13.8 months	Fatigue (2.7%); decreased serum phosphorus (1.4%); increased serum alkaline phosphatase (1.4%)	61
185 patients with advanced-stage *IDH1*-mutant cholangiocarcinoma after ≤2 prior lines of treatment	Ivosidenib (*n* = 124) vs placebo (*n* = 61)	III	**ORR 2.4% vs 0%; SD rate 50.8% vs 27.9%** median PFS 2.7 months vs 1.4 months (HR 0.37, 95% CI 0.25–0.54; *P* < 0.0001); 6-month PFS 32% vs 0%; 12-m PFS 22% vs 0% median OS 10.8 m vs 9.7 m (HR 0.69, 95% CI 0.44–1.10; *P* = 0.06)	Hypophosphataemia (1.7%); fatigue (1.7%); anaemia (0.8%)	62
21 patients with advanced-stage *IDH1*-mutant chondrosarcoma	Ivosidenib	I	**SD rate 52.4%; median PFS 5.6 months; 6-month PFS 39.5%**	Hypophosphataemia (4.8%)	63
66 patients with *IDH1*-mutant glioma that had recurred after or not responded to initial surgery, radiation or chemotherapy	Ivosidenib	I	ORR 2.9% among 35 patients with non-enhancing gliomas and 0% in those with enhancing lesions; SD rate 66.7% overall; median PFS 13.6 months and 1.4 months in patients with non-enhancing and those with enhancing gliomas, respectively	2 patients had grade ≥3 TRAEs (neutropenia, decreased weight, hyponatraemia and/or arthralgia); grade ≥3 treatment-emergent events in the dose-expansion cohort included seizure (4.0%), hypophosphataemia (4.0%), headache (2.0%) and hyperglycaemia (2.0%)	64
33 patients with *IDH1*-R132H-mutant newly diagnosed WHO grade III or IV astrocytoma	20-mer IDH1-R132H peptide vaccine	I	ORR (stable disease) 84.4%; 63% free from progression at 3 years; 84% alive at 3 years	None	65

AML, acute myeloid leukaemia; CR, complete remission/response; EFS, event-free survival; MDS, myelodysplastic syndrome; ORR, objective response rate; OS, overall survival; PFS, progression-free survival; R/R relapsed and/or refractory; SD, stable disease; TRAEs, treatment-related adverse events. [a]In the primary efficacy population comprising 125 patients with R/R disease receiving 500 mg of ivosidenib daily with at least 6 months of follow-up data. [b]Among 179 patients with R/R disease. [c]In 18 evaluable patients across three treatment groups. [d]Among 214 patients with R/R AML receiving 100 mg of enasidenib daily.

1.4 IDH mutations in chondrosarcoma

In a genetic screen of 1200 mesenchymal tumors, including 220 cartilaginous tumors, 222 osteosarcomas and 750 other bone and soft tissue tumors for IDH1R132 with Sequenom mass spectrometry, IDH mutations were identified in 56% of 137 central and periosteal cartilaginous tumours but not in other tumour types. The IDH mutations were predominantly IDH1-R132C (~40%), followed by IDH1-R132G and IDH1-R132H mutations. Cartilaginous tumors and chondroblastic osteosarcoma IDH1wt have been analysed for IDH2 R172 and R140. IDH mutation were prevalent in acral bones (90%), followed by long bones (53%) and flat bones (35%) ($p < .001$). 52% of central low grade tumors were mutant, 59% G2-3 conventional central

chondrosarcomas, and 56% dedifferentiated chondrosarcoma harbored IDH1/2 mutation. Second neoplasm in 1 pts carried the same IDH1 R 132C mutation [26].

IDH prognostic role is controversial. Lugowska et al [67] found worse survival in IDHmut chondrosarcoma, but all patients of that study had peripheral chondrosarcoma, while in the series by Zhou et al, reporting on 89 central chondrosarcoma (51 G2-3), patients with IDH mutant tumors had longer relapse-free survival [68]. Cleven could not find differences in disease specific survival and metastasis free survival between IDH mut and IDH wt conventional central chondrosarcoma (CBCC) of 63 patients [69], nor Amary [26] in the survival period of 50 patients with high grade CBCC. Other authors reported on outcome of patients with IDH mutated chondrosarcoma [70-72], and a meta-analysis using individual patient data from 14 studies found a significant negative impact of IDH1-2 mutations on patients overall survival, but not on RFS and MFS [73]. However, a limitation of most of these studies is the inclusion of patients with different histological types and different histological grade chondrosarcoma. In addition, some of these studies are multicentric.

First aim of this project is to assess the frequency of IDH mutations, their relationship with clinical characteristics, and their prognostic role in patients with high grade CBCC treated in the same Center.

2. IDH mutations in high grade central conventional bone chondrosarcomas

2.1 Matherials and Methods

2.1.1 Study design

Aim of the study was to describe frequency and type of IDH mutations in G2 and G3 CBCC and identify correlations with clinical characteristics and outcome. Inclusion criteria: surgery of primitive tumor at IRCCS Istituto Ortopedico Rizzoli from 2002 to 2012, confirmed diagnosis of G2 or G3 conventional chondrosarcoma, availability of both paraffin-embedded and fresh frozen tissue at the Musculoskeletal Tumor Biobank – BIOTUM. A comprehensive written informed consent was signed for the surgical procedure and related diagnostic procedures in accordance with the standard institutional procedure. After Ethical Institutional Committee approval, clinical data on the diagnosis, treatments and follow-up (according institutional guidelines) were collected retrospectively from the patient charts. Surgical margins were defined according to the Enneking score system [34].

Molecular analyses were performed on formalin-fixed and paraffin-embedded tissues (FFPET) and/or frozen tumor samples of patients included in the study. The sample derived from the first surgical procedure of chondrosarcoma removal performed at Rizzoli. All information regarding the human material used in this study was managed using anonymous numerical codes, clinical data were not used and samples were handled in compliance with the Helsinki declaration.

2.1.2 DNA extraction

Total DNA extraction from FFPET samples was performed using the QIAamp FFPE Tissue kit (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions. A representative tumor area was selected by Multi-Purpose Sampling Tool (Harris UNI-CORE, TedPella Inc, USA) that allows to take tissue cores from the sample, 15andomize DNA from two cores for each sample. With some modifications the same QIAamp FFPE Tissue kit (Qiagen) was used for DNA purification of frozen samples. In brief, a fragment of less than 10mg tumor tissue was selected using scalpel blades. For each sample 180µl of lysis buffer and 20µl of Proteinase K Solution were added. After mechanical stirring, samples were incubated in a water bath over night at

56°C. Hematoxylin-eosin cryostat sections were performed to evaluate if the area of tissue selected was representative of the tumor. Quantitative and qualitative analysis was carried out on DNA obtained from both FFPET and frozen tissue by measuring the absorbance (A) in a spectrophotometer to determine the concentration and purity. DNA was considered suitable for molecular analysis only if the A260 / A280 ratio was greater than 1.8.

2.1.3 Sequencing of Polymerase Chain Reaction products

For PCR reactions, >60 ng of DNA was amplified using AmpliTaq Gold 360 Master Mix (Applied Biosystems, Foster City, CA, USA) with 0.5 μM of primers. Thermal cycling conditions were 95°C for 5 min followed by 40 cycles of 94°C for 45 sec, 58°C for 45 sec and 72°C for 45 sec followed by extension at 72°C for 7 min. The primers used for PCR and sequencing were: IDH1 (forward: 5'-CACCATACGAAATATTCTGG-3'; reverse: 5' CAACATGACTTACTTGATCC-3'); IDH2 (forward: 5'-GCTGCAGTGGGACCACTATT-3'; reverse: 5'-GTGCCCAGGTCAGTGGAT-3'). The sequencing was performed by Bio-Research Fab (www.biofabresearch.it, Rome, Italy). Mutation analysis was conducted with Basic Local Alignment Search Tool (BLAST), in the NCBI database "National Center of Biotechnology Information Database" (http: //www.ncbi. nlm.nih.gov/BLAST). Electropherograms were exported to fasta format and were aligned to the NCBI BLAST sequences.

2.1.4 Statistical analysis

Continuous variables were summarized as mean and range; discrete and categorical variables were summarized using frequencies and percentages. Patient characteristics at diagnosis (age, site, grade, margins according to Enneking [79], and stage), were compared between IDH mutation using Chi-Square test. OS was defined as the time from the date of surgery to the date of death. Patients who did not experience the outcome of interest were censored at the time of last follow-up. RFS was calculated in localized patients and was defined as the time from date of surgery to the date of first relapse. PFS was calculated in metastatic patients and was defined as the time from date of surgery to the date of progression. Patients who died for surgery complications were excluded from survival analysis. Kaplan-Meier methods were used to

estimate OS, RFS and PFS and the curves were compared using log-rank test. A value of $p<0.05$ was considered statistically significant. All p values were 2-sided. Data were analyzed using the SAS 9.4 software (SAS Institute).

2.2 Results

2.2.1 Frequency and type of mutations

DNA extraction was performed on tumor samples derived from the first surgical procedure performed at Rizzoli of the 64 patients included in the protocol.

Molecular analysis was not feasible overall in 10 patients: in 15 cases due to decalcification and aged slides (FFPET) and in 9 cases for limited tissue availability (fresh tissue).

In 54/64 (84%) cases the quality of tissue was adequate: IDH mutations were detected in 24 patients (44%): 18 IDH1mut only (34%), 4 IDH2mut only (7%), and 2 patients both IDH1 and 2mut (3%). IDH1 was mutated on 132 residue of arginine while IDH2 on residue 172, in the substrate-binding site. Details on mutation types are described in Table 3 and Figure 3.

TABLE 3. Distribution of IDH1 and IDH2 gene mutations in 24 patients conventional central bone chondrosarcoma.

Gene	Mutation	Prevalence
IDH1	R132C (tgt)	63% (15/24)
IDH1	R132G (ggt)	17% (4/24)
IDH1	R132S (agt)	4% (1/24)
IDH2	R172S (agt)	17% (4/24)
IDH2	R172S (agc)	4% (1/24)
IDH2	R172G (ggg)	4% (1/24)

Figure 3: Sanger sequencing performed on **IDH1 gene**, exon 4 (FIG 3A) and **IDH 2**, gene exon 4 (FIG 3B)

FIG 3A: (A) Electropherogram data from a wild-type sequence in one of the tested sample; (B) chondrosarcoma sample harboring a R132C IDH1 (arrow) mutation (c.394 C> T); (C) chondrosarcoma sample harboring R132G (arrow)mutation (c.394 C>G); (D) sample harboring R132S (arrow) mutation (c.394 C>A).

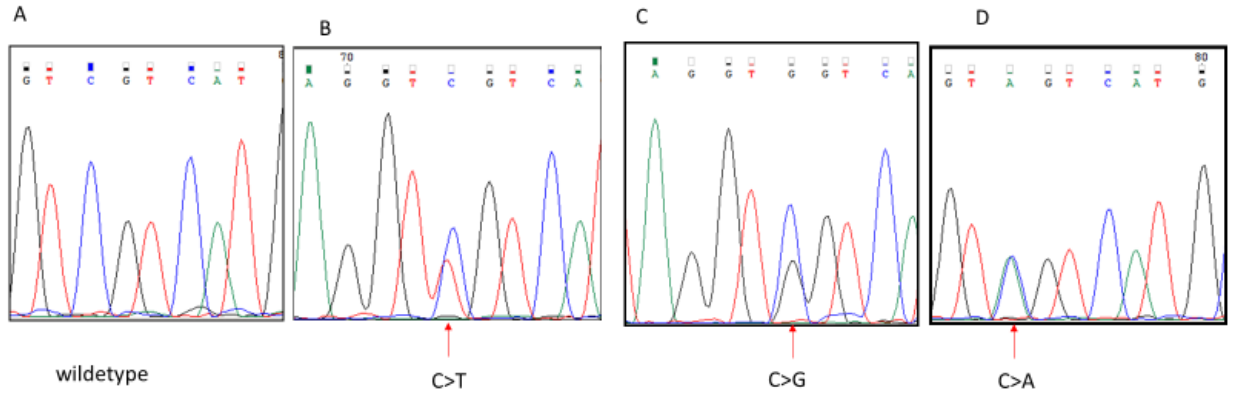
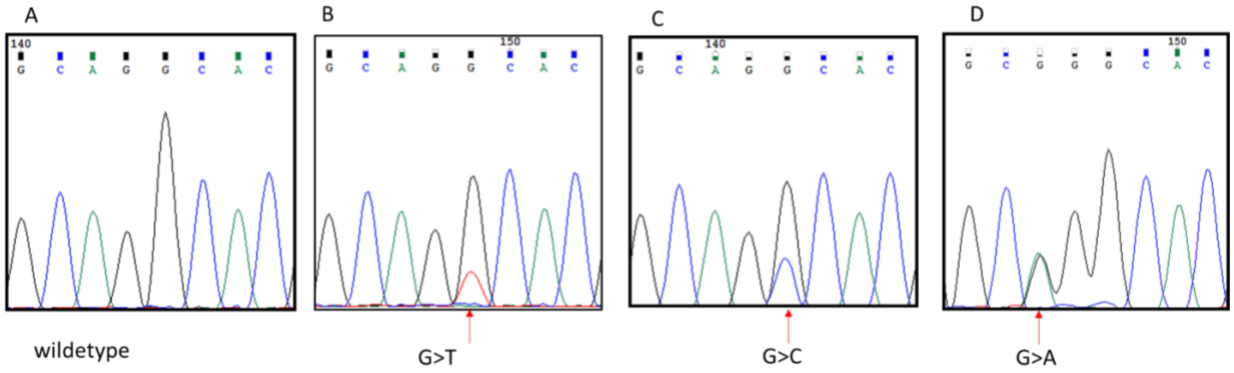


FIG 3B: (A) Electropherogram data from a wild-type sequence in one of the tested sample; (B) chondrosarcoma sample harboring a R172S (arrow) mutation (c.515 G>A); (C) chondrosarcoma sample harboring R172S (arrow) mutation (c.515 G>C); (D) sample harboring R172G (arrow) mutation (c.515 G>A).



2.2.2 Patients clinical characteristics by mutational status

Of the 54 analyzed patients 18 (33%) were male and 36 (67%) female; median age was 63 (range 17-85 years).

At the time of surgery 40 patients (74%) presented with localized disease, and 14 (26%) with lung metastases. 41 patients had G2 chondrosarcoma at pathological examination, while 13 had G3 tumor. 8 (19%) G2 patients, and 6 (42%) G3 patients presented with metastases.

Comparison of clinical characteristics between mutated and wild-type cohorts is illustrated in Table 4. Median age was comparable in mutated and wild-type groups (60 vs. 64 years-old). The IDH1/2 mutation rate was not different in localized (43%) vs. metastatic (50%) patients; while it was significantly higher in G3 (69%) than in G2 (37%) tumors (p=0.0390).

Histology was reviewed by our pathologist: no different morphological features have been reported for mutated versus wild- type tumors.

TABLE 4: Patient characteristics according to IDH mutation splitted by grade

	G2 (N=41)				G3 (N=13)			
	IDHmut N=15 (37%)	**IDHwt** N=26 (63%)	**All G2**	**P value**	**IDHmut** N=9 (69%)	**IDHwt** N=4 (31%)	**All G3**	**P value**
Site, n (%)								
Extremity	10 (67)	10 (38)	20	0.23	4 (44)	1 (25)	5	0.44
Pelvis	3 (20)	10 (38)	13		4 (44)	1 (25)	5	
Other	2 (13)	6 (24)	8		1 (12)	2 (50)	3	
Stage, n (%)								
Localized	12 (80)	21 (81)	33	1.0	5 (56)	2 (50)	7	1.0
Metastatic	3 (20)	5 (19)	8		4 (44)	2 (50)	6	
Margins, n (%)								
Adequate	10 (67)	20 (83)	30	0.27	7 (78)	2 (50)	9	0.53
Inadequate	5 (33)	4 (17)	9		2 (22)	2 (50)	4	
Uk		2	2					

As expected, syndromic conditions have been confirmed to be a substrate for IDH mutated conventional chondrosarcoma. The three chondrosarcomas of patients with Ollier syndrome (100%) harbored a mutation of IDH1and all 3 cases shared the same mutation: R132C(tgt); none of the patients presented IDH2 mutation. One patient with Maffucci syndrome had a metacarpal chondrosarcoma IDH1wt and IDH2 not evaluable.

As shown in Table 4, comparing patients with grade 2 versus grade 3 CCBC, no differences have been found between patients with IDH1/2 mutated and not mutated tumors in terms of localization, stage at diagnosis, morphology and Syndrome coexistence.

2.2.3 Evolution/dedifferentiation

We observed a different grade progression over time (at relapse) in the 2 groups (IDH1/2mut or wt): IDHmut chondrosarcoma had a higher rate of grade progression at relapse, as compared to IDHwt. In particular: 25 patients (21 cases presenting with G2 at diagnosis and 4 presenting with G3) had a new histologic evaluation at recurrence/progression: 5/9 (55%) of G2 IDH1/2mut tumors became at higher grade at recurrence (1 became G3 and 4 dedifferentiated); by contrast only 3/12 (25%) of G2 IDH1/2wt acquired an higher grade in the recurrence (3 became dedifferentiated).

02/30 Two patients that were IDH1wt developed second malignancies: one a desmoid fibromatosis (and had a previous prostatic adenocarcinoma) and one a Non Hodgkin Lymphoma. 2/24 patients with IDHmut developed a second malignancy: 1 patient with a prostatic adenocarcinoma and 1 patient with Maffucci Syndrome developed a second chondrosarcoma. Three patients with Ollier disease, IDH mut, had evidence of pre-existing enchondroma. Moreover, one patient carrying IDH1 and IDH2 mutation at the time of local relapse had evidence of enchondroma, G2 chondrosarcoma, and dedifferentiated chondrosarcoma recapitulating in his history all the phases of cancerogenesis.

2.2.4 Treatment

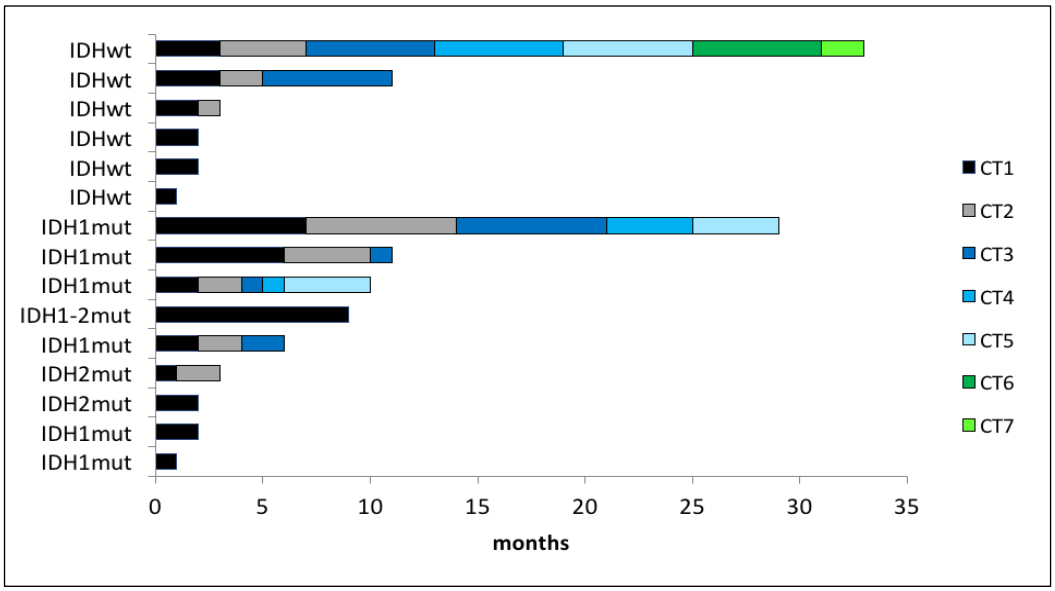
Among 54 patients, all underwent surgery of primary tumor at Rizzoli: 42 resections, 11 amputations and 1 curettage. Wide margins in 39 pts.

None of 40 cases with localized disease received adjuvant treatment.

6/14 patients presenting with metastatic disease at diagnosis (4 IDHmut, 2 IDHwt), underwent first line chemotherapy.

Treatment at recurrence/progression consisted of surgery, chemotherapy, and radiotherapy. 17 underwent surgery, 8 patients underwent radiotherapy, and 9 patients underwent chemotherapy at recurrence/progression. The number and duration of chemotherapy lines are summarized in Figure 4.

FIGURE 4. Chemotherapy lines, for each patient, and their duration.

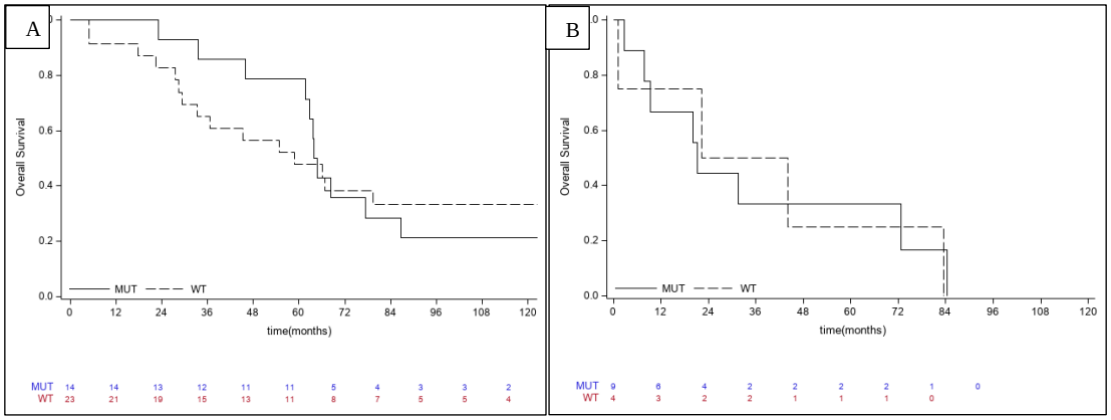


2.2.5 Outcome

Survival analysis was performed in 50 patients: 4 patients died for surgery complications and have been excluded from the outcome analysis. After a median follow-up of 124 months (range 1-166) disease recurrence occurred in 21/40 localized patients (10 mutated): 11 local recurrence, 5 lung metastases, 1 lymph nodes, and 4 both local and distant metastases. Disease progression was reported for all metastatic patients.

The 5-years OS was 51% (95% CI:36-64) and was significantly higher in patients with localized vs. metastatic disease at diagnosis (69% vs. 7%, $p<0.001$), and in patients with grade G2 vs G3 (59% vs. 29%, $p=0.0078$) chondrosarcoma. No difference in survival by mutational status (5-year OS IDHmut 61% vs IDHwt 44%, $p=0.6854$) has been detected. No difference in survival was observed for gender and site. Splitting by grade, 5y OS in G2 was 79% (95% CI: 47-93) for IDHmut vs 48% (95% CI: 27-66) for IDHwt (p 0.85), and in G3 33% (95% CI 8-62) in IDHmut vs 25% (95% CI: 8-67) in IDHwt (Figure 5).

Figure 5: Overall survival according to mutational status in G2 (A) and G3 (B) patients



The 5-year RFS in localized patients was 33% (95% CI:10-57) in IDHmut and 57% (95%CI: 30-77) IDHwt (p=0.3596) (Figure 7).

In patients presenting with metastases, the PFS was 25% (95%CI: 1-65) in IDHmut and 16% (95%CI: 0.7-52) in IDHwt, (p=0.9126) (Figure 8).

2.3 Discussion

IDH mutations were first identified in 2006 in colon cancer [17], and next in many other tumor types, but only in glioma, a clear correlation between genetic alteration and disease phenotype and prognosis has been established [19].

In her pioneering works Amary demonstrated that IDH mutations in mesenchymal tumors: 1- are prevalent in cartilaginous tumors rather than in other connective tissue derived sarcoma; 2- are present in 80% of benign enchondroma and in almost half of conventional chondrosarcoma; 3- occur early in the carcinogenesis; 4- both local and distant recurrences of central conventional chondrosarcoma maintain the same IDH mutational status of the primary lesion 5- are almost always present in enchondroma of Ollier and Maffucci syndromes [26, 74-75].

Other authors investigated the presence and role of IDH mutations in chondrosarcomas, but most of the reported series include benign with high grade tumors from different sites, and also different chondrosarcoma histotypes. In that series the number of G2 and G3 chondrosarcoma

accounts from 24 to 51. [67-73]. In the current study, we decided to focus on central conventional chondrosarcoma G2 and G3 to have a homogeneous population, reporting data on 54 patients all treated in one Institute.

Sanger sequencing has been performed on IDH 1 and 2 genes, and amino acid substitutions at residues R132 in IDH1 and R172 in IDH2, but not at R140 in IDH2, have been detected, which is consistent with previous studies in chondrosarcoma, even if detection technologies and assays are different.

The mutation rate was 44%, with a prevalence of IDH1 mutations (IDH1 to IDH2 mutation ratio 20:6). The frequency of mutations was significantly higher in G3 chondrosarcoma than in G2 (69% vs 37%, $p = 0.039$), which is consistent with literature data. Humerus (100%) and tibia (80%) were the most frequent mutated site, followed by the femur (35%) and pelvis (38%). One vertebral chondrosarcoma was mutated (no other described in the literature). All the 3 patients with Ollier disease carried IDH1 mutation, while the patient with Maffucci syndrome was IDH1wt and IDH2 not evaluable. Individuals affected by these rare congenital non-hereditary disorders, characterized by enchondromatosis, haemangiomatosis, and physical deformities, harbor somatic IDH mutations in 77%-90% of the cases, and transformation of enchondroma to chondrosarcoma occurs in more than 30% of the cases. The high prevalence of IDH mutations in enchondromas and the evidence that recurrent central conventional chondrosarcomas retain the same IDH mutational status of the primary tumor support the hypothesis that IDH mutation is an early event in chondrosarcoma-genesis also in non-syndromic patients. [76].

Chondrosarcoma recurrence is estimated to exhibit a higher grade of malignancy in more than 10% of cases, but biological mechanisms involved in this progression are the object of research [77]. We have examined the pathological grade at recurrence in 25 patients (21 G2 and 4 G3). 55% IDHmut vs 25% IDH wt G2 became higher grade at relapse. One patient carrying IDH1 and IDH2 mutation at the time of local relapse had evidence of pre-existing enchondroma, G2 chondrosarcoma, and developed dedifferentiated chondrosarcoma. Taken together our data suggest that IDH mutation might be one of the events responsible for low grade to high grade/ dedifferentiated chondrosarcoma progression, and IDH mutant cells are more susceptible to changes toward dedifferentiation. For example, p16/CDKN2A copy number variation (gain or loss), which is associated with 75% of high grade central chondrosarcoma, in IDH mut tumors

occurs only after the onset of IDH mutation [76]. Also, the methylation status changes from benign/low grade and high grade IDH mutant chondrosarcoma, and mainly affects signal transduction and inflammation-related genes [78].

Differently from glioma, the impact of IDH mutation on chondrosarcoma prognosis is still not clear. Stage, grade, size, and surgical margins are the main factors to consider for survival prediction in CCBC. 5-years overall survival for grade 2 and 3 lesions is 81% and 29% respectively, and metastatic potential is 10-15% for grade 2 and more than 50% for grade 3 [26]. In our series, 19% of G2 and 43% of G3 had lung metastasis at the time of first surgery in our Institute. Grade and stage confirmed to be prognostic, but not the mutational status. The 5-year OS on 50 pts was 51%, and dropped to 7% in metastatic patients, and to 29% in G3 patients, but did not differ significantly by mutational status. The 5-year RFS was worse in CCBC localized patients, but not statistically significant. Similarly, Zhu et al reported longer relapse-free and metastasis-free survival in patients with IDH mut high-grade chondrosarcomas and did not detect a significant impact of mutational status on overall survival. In contrast Lugowska [67] et al found shorter survival in IDH1/2mut patients, but in that series, different histotypes other than conventional have been included. A recent meta-analysis of 14 published studies concluded that IDH1/2 mutated patients had a higher risk of death but not significantly different RFS and MFS [73].

Surgery is fundamental for chondrosarcoma treatment, repeated procedures are indicated for local recurrences and lung metastases [79,80]. In our series, all patients underwent surgery of the primitive tumor and 17 patients had surgery at relapse. Fifteen patients have been treated with chemotherapy, but only one of them, who received chemotherapy according to Euro-B.O.S.S protocol [81] for a dedifferentiated recurrence, had tumor shrinkage. One patient received adjuvant chemotherapy after dedifferentiated recurrence. The other 13 patients had disease progression after 2-6 months in all chemotherapy lines. The intrinsic chemoresistance in chondrosarcoma is mainly due to dense extracellular matrix, poor vascularization, and a low percentage of dividing cells [82,83]. There is an urgent need to unveil biological mechanisms to find new treatments [84-86]. Five of our patients have been treated with rapamycin and cyclophosphamide since P13-Akt-mTOR is a frequently altered pathway in conventional chondrosarcoma [87-88], but only one achieved a disease stabilization as best response.

Hedgehog inhibitors did not induce a response in two patients even if the hedgehog pathway is known to be upregulated in conventional chondrosarcoma [89]. Other target therapies in chondrosarcoma include antiangiogenic agents, histone deacetylase inhibitors, immunotherapy, and more recently IDH inhibitors [90].

Drugs targeting IDH1 and IDH2 mutated proteins are approved for the treatment of IDH mutated acute myeloid leukemia. Preclinical studies and a phase I clinical trial have been conducted with a selective IDH1 inhibitor [91-95]. Ivosidenib is a first-in-class selective oral IDH1 inhibitor developed by Agios Pharmaceuticals has shown clinical activity in phase 1, oan pen-label, multicentre study with 168 patients with advanced solid tumors (NCT02073994): 11/20 achieved stable disease, 4 for more than 6 months. Ongoing trials with IDH1/2 inhibitors are still recruiting chondrosarcoma patients.

2.4 Conclusions

No different clinical characteristics have been found between IDHwt and IDHmut high-grade conventional chondrosarcoma in our series, except higher frequency of mutations in Syndromic patients and in G3.

No statistically significant difference in prognosis between IDHwt and IDHmut.

A different trend in histologic evolution has been detected, with IDHmut G2 tumors having a higher rate of grade progression at relapse, as compared to that. Confirmation of our results is needed in larger series [96].

3. IDH mutations at the intersection of cellular metabolism and microenvironment

3.1 IDH mutations and cancer metabolism

Many of the biological effects of IDH mutations are independent of the cell or cancer type, including over-production of D-2HG, hypermethylation, and blocks to normal differentiation patterns. Despite these commonalities, major differences exist, among IDHmut cancers in the effects on metabolism and response to therapy, suggesting cancer-dependent, tissue-dependent, and even differentiation state-dependent phenotypes.

In IDH mut cells NADPH production is impaired: by consuming rather than generating NADPH, mutant IDH causes metabolic reprogramming that results in dysregulation of gene expression, DNA damage repair, inflammation, intracellular trafficking, aging and cell death. Noteworthy low basal level of NAD+ in IDH1-mut cells constitute a potential therapeutic liability that can be exploited using various drugs, including temozolomide and poly(ADP-ribose) and polymerase (PARP) inhibitors.

In IDH1-mut tumor xenograft models, the vulnerability linked to low NAD+ levels has been explored through inhibition of nicotinamide phosphoribosyltransferase (NAMPT), the rate-limiting enzyme of the NAD+ synthesis pathway, which resulted in decreased tumor growth and prolonged survival [46].

The discovery of the homologous recombination deficiency associated with IDH mutations led to the screening of a panel of DNA repair pathway inhibitors; IDH1-mutant cells were found to have a 45-fold increase in sensitivity to the PARP inhibitor 26andomiz relative to IDH-wild-type cells [52].

Future studies have to take into account that IDH mutations are probably dependent on the cell type and genetic context, leading to context-dependent prognostic and therapeutic implications [66].

3.2 IDH mutations and differentiation

Mutant IDH-mediated epigenetic dysregulation with subsequent effects on differentiation states

has been observed in different cell types: IDH-mutant mouse hepatoblasts fail to differentiate into hepatocytes owing to D-2HG-mediated silencing of the master transcriptional regulator HNF4α, correlated to reduced H3K4me3.

In a mouse pre-adipocyte 3T3-L1 cell line, expression of mutant IDH causes a defect in the adipogenesis program via downregulation of several transcription factors, including those encoded by Cebpa, Pparg, and Adipoq, blocking adipocyte differentiation.

Similarly, expression of IDH1-R132C in human mesenchymal stem cells results in increased levels of H3K9me3 and H3K27me3 and H3K4me3, with upregulation of several early and late markers of chondrogenic differentiation and downregulation of osteogenic markers. These findings might explain why IDH mutations are prevalent in chondrosarcomas but not in osteosarcomas. Together, the results of these studies implicate histone methylation defects in mutant IDH-mediated impairments in cellular differentiation [66].

3.3 IDH mutation and methylation

The acquisition of hypermethylated state in IDH mut cells is linked to the competitive inhibition, by D-2HG, of the TET family of methylcytosine hydroxylases, a class of α-KG-dependent dioxygenases, which promote DNA demethylation via conversion of 5-methylcytosine (5mC) into 5-hydroxymethylcytosine (5hmC) [99,100].

DNA methylation, which predominantly occurs at CpG islands, has variable effects on gene expression depending on the balance between activating H3K4me3 and repressive H3K27me3.

Guilhamon et al performed a genome wide methylation profiling of 44 central chondrosarcomas, with a meta-analysis of hypermethylation phenotype of AML, low grade glioma, and cholangiocarcinoma. In all these tumor types, a common mechanism has been suggested: IDH mutation is associated with reduced demethylation, through inhibition of the TET family of oxygenases. The analysis shows an overlapping in some of the methylated genes, and other tissue-specific pathways: pathways involving the function of osteoblasts, osteoclasts, and chondrocytes in CS, axonal guidance signaling in LGG, Myc signaling in AML, and circadian rhythm signaling in CC. This suggests a tissue-specific hypermethylation phenotype of each tumour type. In chondrosarcoma, the most significantly affected physiological function category was tissue development, with the development of connective tissue and adhesion of carcinoma

cell lines and fibroblasts as the top functions [101]

3.4 IDH mutation and angiogenesis

Angiogenesis pathways are potentially effective targets for arresting the growth and spread of chondrosarcomas. Chondrosarcomas have been shown to exhibit a microvascularity that has been associated with aggressive clinical behavior and a higher potential for metastasis. In 2011, a study involving 58 conventional chondrosarcomas found that microvessel densities in chondrosarcoma tumors correlate with histological grade and subsequently prognosis, suggestive of a role for neovasculature in the clinical behavior of chondrosarcoma [VEGF (vascular endothelial growth factor) is essential for the neovascularization required to sustain and propagate a tumor. Efforts to develop antiangiogenic therapies have produced many agents including the small molecule tyrosine kinase inhibitor, pazopanib, and full human monoclonal (IgG1) antibodies, such as bevacizumab and ramucirumab, which affect angiogenesis by binding VEGF or the VEGF receptor (VEGFR), respectively [84].

A preclinical study demonstrated that IDH1mut knockout abrogates chondrosarcoma genesis through modulation of integrins, addressing integrin molecules as appealing candidates for combinatorial regimens with IDH1mut inhibitors for chondrosarcomas that harbor this mutation. Moreover, modulation of integrins by IDH mutation appears to be cell type specific. Furthermore, since integrins are known to have an important role in angiogenesis the authors found that vascular density was significantly lower in the IDH1mut knockout tumors compared to the parental control tumors, which could also be responsible for the slower growth of these tumors. Nonetheless, the study supports the notion that IDH mutations promote chondrosarcoma growth through the modulation of integrins [102].

3.5 IDH mutation and tumor immunity

In one study including a large cohort of chondrosarcoma patients, 41% of the dedifferentiated chondrosarcomas had immunohistochemistry positivity for PD-L1, in association with HLA class I expression and a high T-cell infiltrate, reflecting an immune-active microenvironment, especially in the dedifferentiated area of the tumor, indicating these patients as a possible candidate to PD-1/PD-L1-targeted therapies. In that series, information regarding IDH mutation

status was available for 10 tumors. The presence of an IDH mutation was not associated with the expression of PD-L1 or a specific immune profile (HLA class I expression, amount of T-cell, and macrophage infiltrate) [103].

A recent study was conducted integrating multidimensional analyses involving genetic factors, cytology, pathology, and clinical parameters, to better characterize the chondrosarcoma immune microenvironment to understand better which patients could benefit most of the immunotherapeutic approaches [104]. 98 newly diagnosed chondrosarcomas were analyzed integrating single-cell CyTOF, whole-exome sequencing, and flow cytometry, leading to the identification of 3 different immune microenvironment phenotypes: subtype I, the "G-MDSC dominant" cluster, with a high number of HLA-DR-CD14- myeloid cells; subtype II, the "immune exhausted" cluster, with high exhausted T-cell and dendritic cell infiltration; subtype III, the "immune desert" cluster, with few immune cells.

While subtype III was characterized by mixed transformation, subtype I and II were immune cell-rich and were characterized by IDH mutation, pathological high grade, and peritumoral edema. 3 patients who responded to anti PD-1 antibody immunotherapy, belonged to the subtype II group ("immune exhausted").

IDH mutation was associated with immune response and chemokine levels in chondrosarcoma.

Whole exome sequencing of 22 samples detected 50% of IDH mutant chondrosarcomas: 8 IDH1 and 3 IDH2.

IDH1/2 mut chondrosarcomas had a higher amount of immune cell subpopulations in comparison to IDH1/2 wt: T-cells, antigen-presenting cDCs, and G-MDSCs. Chemokines evaluation revealed a more inflamed microenvironment in IDH1/2-mut, with a significantly higher level of CXCL9, CXCL12, and CXCL10. These chemokines attract CXCR3+ and CXCR4+ immune cells.

A high D-2-HG concentration in IDH1/2- 3 mut cases was correlated with cDCs and CXCL12 levels.

These data clearly show that IDH mutation is a potential marker for response to immunotherapy in chondrosarcoma.

Clinical features significantly associated with higher immune cells infiltration were: tumor grade, peritumoral edema in MRI, and myxoid transformation. High-grade chondrosarcomas had

significantly more multiple immune cell subtypes, especially CD8+ 3T-cells and cDCs. The presence of IDH mutation associated with edema in myxoid-free high-grade chondrosarcoma could predict the presence of an immune exhausted microenvironment.

Further study on CXCL12 in bone sarcomas is warranted since its importance is also in osteosarcoma and chondroblastoma. However immune microenvironment of chondrosarcoma is different from osteosarcoma and chondroblastoma, with a low amount of TAMs, so therapies TAMs are not encouraged in chondrosarcoma. Thus, therapeutics targeting, such as immune checkpoint inhibitors (ICIs) are promising for this chondrosarcoma, since they target exhausted T-cells. IDH mutation may be a vital role in mediating immune response in CHS by facilitating DC homing through the interaction with CXCL12 [104].

In conclusion, great progress has been made in understanding the biology of IDH mutations in a variety of cancers and their pathogenic roles are beginning to be elucidated. These hotspot mutations remain a promising therapeutic target, and understanding their biological effects in the different cancer types particular is crucial to their successful clinical translation.

4. IDH mutations in soft tissue sarcomas

4.1 Sequencing analysis

The sequencing analysis, through whole-exome sequencing (WES) and whole transcriptome sequencing (WTS), has been performed on tumor samples and peripheral blood of 15 patients gastrointestinal stromal tumors (GIST) and 5 rare soft tissue sarcomas (STS).

DNA was extracted from peripheral blood and fresh frozen tissue with a DNA mini kit (Qiagen, Milan, Italy) and through QiAmp DNA micro kit (Qiagen) from formalin-fixed and paraffin-embedded tissues (FFPE) following the manufacturer's instructions. WES of tumor DNA was performed on the HiScanSQ platform by Nextera Rapid Exome Enrichment protocol (Illumina, San Diego, California, USA). Briefly, 100 ng of genomic DNA was tagged and fragmented by the Nextera transposon. The Nextera transposome simultaneously fragments the genomic DNA and adds adapter sequences to the ends. The products were then amplified and exome regions were enriched. The enriched libraries were amplified by PCR and quantified using PicoGreen assay (Life Technologies, Milan, Italy).

For WTS, Rneasy Mini Kit (Qiagen, Milan, Italy) was employed to extract the total RNA from tumor specimens. For the RNA-seq samples, the cDNA libraries were synthesized starting from 250 ng total RNA with TruSeq RNA Sample Prep Kit v2 (Illumina, San Diego, CA, USA) following the manufacturer's protocol. HiScanSQ sequencer (Illumina) was used to generate sequences at 75bp in paired-end mode yielding an average of 61 million mapped reads/sample, reaching an average coverage of 44X.

4.2 Bioinformatic analysis

Bioinformatic analysis of genomic data derived from WES and WTS has been performed to analyze IDH1 and IDH2 gene variants in both populations.

4.3 Results

4.3.1 Rare soft tissue sarcomas

Through RNA sequencing of STS (15 extraskeletal myxoid chondrosarcomas, 13 dermatofibrosarcoma protuberans, and 13 myoepiteliomas) IDH mutations have been identified in 3 cases. These are uncertain significance variants since the germinal data is lacking.

4.3.2 GIST

Exome Sequencing has been performed on 35 cases, among which 7 are of interest.

RNA Sequencing from FFPET has been performed on 30 cases, 18 of interest.

Among the IDH mutated, 3 cases have been studied with both WES and WTS. In that cases the quantitive data of gene expression in CPM (count per million reads) is indicated in TABLE :

Tumor	Gene	Isoform	Exon	cDNA	Protein	Somatic/Germline	IDH1 CPM	IDH2 CPM
GIST Q-	IDH1	NM_005896	exon6	c.A548G	p.Y183C	GERMLINE	112.3	85.7
GIST Q-	IDH2	NM_002168	exon5	c.C588G	p.F196L	GERMLINE	88.3	65
GIST KIT-mutant	IDH2	NM_002168	exon6	c.G782A	p.R261H	GERMLINE	59.8	34.7

WES: we have identified 2 GIST with potentially pathogenic somatic variants (1 IDH1 and 1 IDH2) not present in the normal counterpart. In the other cases some genetic variants have been detected, but, since the normal control is lacking, no conclusive data can be drawn.

WTS: we have identified genetic variants in 18 cases, but, since is an RNA method, it's possible that this data could be not reliable.

TABLE A

Tumor	Gene	Isoform	Exon	cDNA	Protein	chr	position hg19	ref base	alt base	Somatic/ Germline	Note
EMCS NR4A3-EWSR1	IDH1	IDH1	NM_005896	exon6	c.A538G	p.M180V	2	208243587	T	C	UNKNOWN
EMCS NR4A3-EWSR1	IDH1	IDH1	NM_005896	exon6	c.G532A	p.V178I	2	208243593	C	T	UNKNOWN
EMCS NR4A3-TAF15	IDH1	IDH1	NM_005896	exon6	c.G532A	p.V178I	2	208243593	C	T	UNKNOWN

TABLE A: 3 cases of extraskeletal myxoid chondrosarcoma with uncertain significant variants of IDH1 mutations

TABLE B

Tumor	Gene	Isoform	Exon	cDNA	Protein	chr	position hg38	ref base	alt base
GIST Q-	IDH1	NM_005896	exon6	c.G532A	p.V178I	2	208243593	C	T
GIST NF1	IDH2	NM_001289910	exon3	c.T212G	p.V71G	15	90090484	A	C
GIST Q-	IDH1	NM_005896	exon5	c.T494C	p.V165A	2	208245345	A	G
GIST Q-	IDH2	NM_001289910	exon7	c.C661T	p.H221Y	15	90087262	G	A
GIST SDH-deficient	IDH2	NM_001289910	exon6	c.A659G	p.K220R	15	90087439	T	C
GIST SDH-deficient	IDH2	NM_001289910	exon3	c.A215C	p.E72A	15	90090481	T	G
GIST SDH-deficient	IDH1	NM_001282387	exon5	c.A518C	p.E173A	2	208245321	T	G
GIST KIT-mutant	IDH1	NM_005896	exon6	c.G532A	p.V178I	2	208243593	C	T
GIST Q-	IDH2	NM_001289910	exon9	c.T1019A	p.I340N	15	90085004	A	T
GIST Q-	IDH1	NM_005896	exon3	c.A72T	p.E24D	2	208251480	T	A
GIST KIT-mutant	IDH1	NM_005896	exon3	c.A13T	p.I5F	2	208251539	T	A
GIST KIT-mutant	IDH1	NM_005896	exon6	c.C580T	p.H194Y	2	208243545	G	A
GIST Q-	IDH2	NM_001289910	exon11	c.C1145T	p.T382I	15	90084324	G	A
GIST Q-	IDH1	NM_005896	exon8	c.C932T	p.T311I	2	208239922	G	A
GIST KIT-mutant	IDH2	NM_001289910	exon6	c.A659G	p.K220R	15	90087439	T	C
GIST KIT-mutant	IDH1	NM_005896	exon6	c.G532A	p.V178I	2	208243593	C	T
GIST Q-	IDH2	NM_001289910	exon11	c.C1163T	p.T388I	15	90084306	G	A
GIST Q-	IDH2	NM_001289910	exon5	c.C436T	p.P146S	15	90088445	G	A

TABLE B: RNA sequencing of 18/30 GIST: identified variants are of low confidence.

TABLE C

Tumor	Gene	Isoform	Exon	cDNA	Protein	chr	position hg19	ref base	alt base	Somatic/Germ line
GIST Q-	IDH1	NM_005896	exon6	c.A548G	p.Y183C	2	209108301	T	C	GERMLINE
GIST Q-	IDH2	NM_002168	exon5	c.C588G	p.F196L	15	90631681	G	C	GERMLINE
GIST KIT-mutant	IDH2	NM_002168	exon2	c.C178T	p.R60C	15	90634814	G	A	UNKNOWN
GIST KIT-mutant	IDH2	NM_002168	exon6	c.G782A	p.R261H	15	90630704	C	T	UNKNOWN
GIST KIT-mutant	IDH2	NM_002168	exon6	c.G782A	p.R261H	15	90630704	C	T	GERMLINE
GIST KIT-mutant	**IDH2**	**NM_002168**	**exon11**	**c.G1340A**	**p.R447K**	**15**	**90084285**	**C**	**T**	**SOMATIC**
GIST PDGFRA-mutant	**IDH1**	**NM_005896**	**exon7**	**c.A815G**	**p.Y272C**	**2**	**209106753**	**T**	**C**	**SOMATIC**

TABLE C Whole exome sequencing of 18/30 GIST. The red cases carried a potentially pathogenic mutation.

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6. ACKNOWLEDGMENTS

I thank:
Prof. Pierluigi Lollini and Prof. Manuela Ferracin, PhD chairs, for their support in the good and bad times of my PhD.
My supervisors, Prof. Maria Abbondanza Pantaleo, and Prof. Nicola Baldini, Prof. Davide Maria Donati.
Dr. Laura Campanacci, Dr. Stefano Ferrari, and Associazione Mario Campanacci.
Dr. Alessandra Longhi.
Alberto Righi, for sharing ideas.
Stefania Benini, Gabriella Gamberi, Giovanna and Stefy, Marco Gambarotti, Cristina Ferrari, Michela Pierini and Elisa Carretta.
Emanuela Palmerini, for our "white bar" early morning meetings and for inspiring my research with her example of perseverance and passion.
Paola Feraco, for being open to scientific collaboration and friendship.
My colleagues Marilena Cesari, Anna Paioli and Rossella Hakim.
My patients for giving me everyday the reason for research.
Lorenzina, Ale, Paola and Cristiana for everything.
Micky for the academic corrections.
My family for good advices, and all my friends!