THE MENISCUS: BASIC SCIENCE TO IMPROVE KNOWLEDGE FOR TISSUE ENGINEERING

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1. Introduction

1.1. Focus on meniscus

Menisci are fibro-cartilaginous structures interposed between femoral and tibial condyles within the knee synovial joint [Melrose et al. 2005; Gamer et al. 2006; McDermott et al. 2008; Rattner et al. 2011; Di Giancamillo et al. 2014; Deponti et al. 2015; Peretti, 2019] (Fig. 1).

For long time menisci were considered only as vestigial structure in the knee [Sutton, 1897; Rai et McNulty,2017]. However, their fundamental role in the bio-mechanics and physiology of the knee joint has been widely recognised in the last years. Menisci are involved in load transmission across the joint (see Chapter 1.7 [Fairbank, 1948; Walker and Erkman, 1975; Seedhom, 1976; Seedhom and Hargreaves, 1979; Fukubayashi and Kurosawa, 1980; Aspden et al., 1985; Arnoczky et al., 1987; and reviewed by Fox et al. 2015]), shock absorption [Krause et al., 1976; Kurosawa et al., 1980; Voloshin and Wosk, 1983; Arnoczky et al., 1987; Fithian et al., 1990; and reviewed by Fox et al. 2015], nutrient distribution [Bird and Sweet, 1987, 1988; Renstrom and Johnson, 1990; and reviewed by Fox et al. 2015], joint lubrication [Macconaill, 1932, 1946, 1950; Renstrom and Johnson, 1990; and reviewed by Fox et al. 2015] and stability [Markolf et al., 1976; Fukubayashi et al., 1982; Levy et al., 1982, 1989; Ahmed and Burke, 1983; Shoemaker and Markolf, 1986; Freutel et al., 1992; and reviewed by Fox et al. 2015] and they also showed a role in proprioception [Wilson et al., 1969; Zimny et al., 1988; Assimakopoulos et al., 1992; Jerosch et al., 1996; Messner and Gao, 1998; Gray, 1999; Saygi et al., 2005; Akgun et al., 2008; Karahan et al., 2010; and as reviewed by Fox et al. 2015) and nociception [Messner and Gao, 1998; Gray, 1999; and reviewed by Fox et al., 2015].

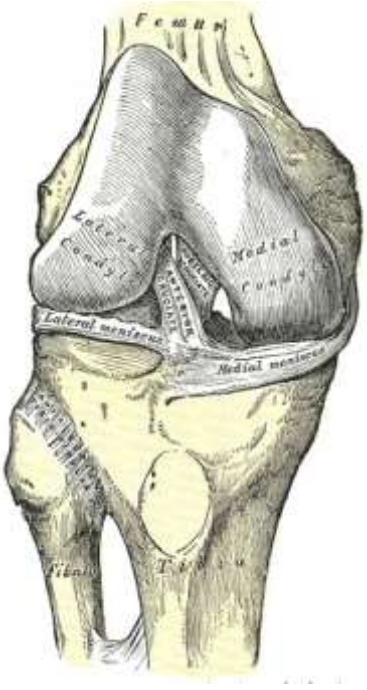

Fig. 1 *Drawing of knee structures, menisci included. From Gray, Henry. Anatomy of the Human Body. Philadelphia: Lea & Febiger, 1918; Bartleby.com, 2000. www.bartleby.com/107/.*

The wedge shape cross-section of menisci (see chapter 1.2) allows them to improve the contact surfaces between the two articular heads of the knee: due to their peculiar shape they increase the congruence between the round femoral condyles and the flatten tibial plateau [Kettelkamp and Jacobs, 1972; Walker and Erkman, 1975; Di Giancamillo et al. 2017; Peretti et al., 2019].

The impact of meniscus upon intraarticular wellness of knee joint is well explained by the demonstrating higher incidence of degenerative changes within the joint (such as osteoarthritis) consequent to its injury or removal [Vaquero and Forriol, 2016]. It was reported that approximately 50% of human patients with meniscus injury developed knee osteoarthritis within 10 to 20 years from the injury [Lohmander et al. 2007; Starke et al. 2009; Yang et al. 2019]. Logerstedt and colleagues (2010) reported an incidence of 12% to 14% and a prevalence of 61 cases per 100.000 persons of meniscal injuries which makes this pathology the second most common injury to the human knee. A high incidence of meniscal tears is reported to be secondary to an anterior cruciate ligament (ACL) injury in the 22% to 86% of cases [O'Connor et al., 2005]. In humans, meniscal tears are the most common pathology that affected intraarticular knee structures [Morgan et al., 1991; Garrett et al. 2006; McDermott, 2006; Englund et al., 2008; Salata et al. 2010; and as reviewed by Ridley et al., 2017].

Englund et al. reported that 19% of the 50-59 years old women and 56% of the 70-90 years old men were affected by meniscal injuries [Englund et al. 2008].

Few studies are focused on the prevalence of meniscal tears in younger population.

Yang et al. (2019) reviewed the incidence of meniscal injuries in the adolescent population and reported a mean value of 52 per 100,000 patients, which is almost half to that of patients 20–50 years old [Yang et al. 2019]. The incidence of meniscal injury in young athletes have been reported as high as 40.7 per 100,000 in males and 22.3 per 100,000 in females [Mitchell et al., 2016]. While, meniscus injuries in patients up to 9 years old are rare with 1 meniscus injury per 100,000 patients [Hede et al., 1990].

Meniscal tears are reported as spontaneous or, more frequently, secondary to ligaments deficiency lesions even in the canine species and, seen the gross anatomy similarity between human and canine menisci, this causes the choice of this species as one of the most utilized animal models in meniscal pathology and reparation [Krupkova et al. 2018].

Reparation of menisci may occur via two different mechanisms following an extrinsic or an intrinsic pathway [as reviewed by Vaquero and Forriol, 2016].

The extrinsic path used a population of undifferentiated mesenchymal cells and nutrient delivered by a pre-existed capillary network (this mechanism is characteristic of the outer/vascular area of the meniscus; see chapter 1.4) [as reviewed by Vaquero and Forriol, 2016]. The intrinsic pathway is based only on the self-repair ability of fibro-cartilage and synovial fluid [as reviewed by Vaquero and Forriol, 2016].

Meniscal healing is strictly linked to the restoration of its mechanical properties and this could be easier for injuries that affect the outer (vascular) area and with a longitudinal direction than those that affect

the inner (avascular) area and with a radial direction, where the restoration of a satisfactory mechanical function is hardly reached by the currently available repair techniques [Vaquero and Ferriol, 2016], the underlying reasons for this will be explained in chapter 1.6.

Nevertheless, the current repair techniques are mechanically superior to partial meniscectomy [as reviewed by Vaquero and Ferriol, 2016]. Meniscectomy, i.e. the complete or partial removal of the damaged meniscus or of its parts, is the current primary method to treat a meniscal injury [as reviewed by Guo et al. 2014] and it has been associated with the precocious insurgence of knee osteoarthritis already in the 1948 [Fairbank, 1948] and, since then, numerous attempts had been tried to obtain the anatomical and functional restoration of this structure via other techniques that lead to the complete restoration of meniscal function.

Nowadays, different techniques are available to achieve the reparation (through sutures or implants) or the complete replacement (with allograft or scaffolds) of the meniscus [Vaquero and Ferriol, 2016].

Nonetheless, tears that affect the avascular inner zone typically fail to heal [King, 1990; as reviewed by Vanderploeg et al., 2012] and are often not eligible for the current surgical repair techniques [Boyd and Myers, 2003; as reviewd by Vanderploeg et al 2012], and, when surgical repair is attempted, these injuries heal with mechanically inferior tissue, which is prone to re-injury [Roeddecker et al., 1994; as reviewed by Vanderploeg et al 2012].

Full or partial meniscectomies are the only alternative in these cases (Fig. 2) [Mordecai et al. 2014].

However, it has been demonstrated as the advantages of meniscectomy cover only a short-term period [Guo et al. 2014] and often it results in the early onset of osteoarthritis (Roos et al. 1995, 1998; Cicuttini et al. 2002; and as reviewed by Vanderploeg et al 2012). Therefore, this procedure should be considered even more as a last-chance option. Unfortunately, the current surgical alternatives to meniscectomy lack

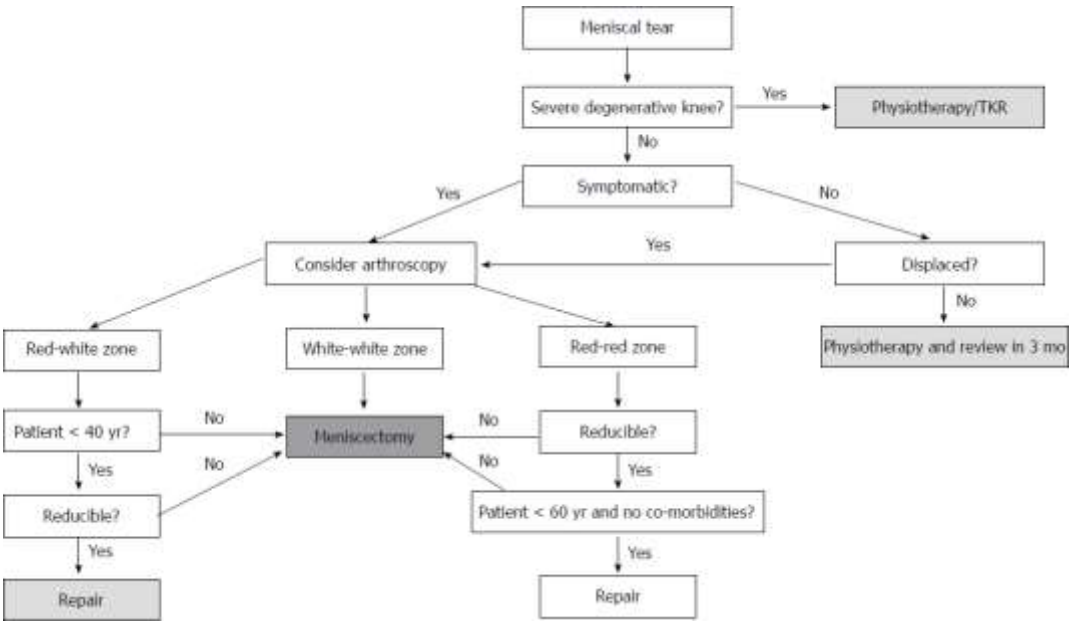

Fig. 2 Meniscal tear management tree. From Mordecai et al. 2014

of long-term evaluation of outcome and suffer from the subsequent degeneration of knee joints, the limited donor organs and the incapacity to entirely mimic the mechanical and biological structure of the native meniscus [Vaquero and Forriol, 2016; Sun et al. 2017].

The crucial role of menisci within the knee joint and its consequential high risk of involvement in knee pathologies are well demonstrated and explicate by the numerous attempts to recover this structure that have been tried during the last years. However, a definitive solution to meniscal tear is still lacking, this may be due in large part due to an incomplete understanding of the complex structure–function relationships that exist in normal menisci [Rattner et al., 2011] and to a still incomplete knowledge of meniscus components behaviour under physiologic and pathologic conditions. Consequently, further studies in these fields are needed to fill these still existing gaps.

For all the previously described reasons, whoever would like to deal with meniscal healing process or techniques must consider how the meniscus' anatomic structure is tightly interconnected to its function and how this may affect the possible further therapeutic outcomes and regenerative medicine attempts.

1.2. Anatomy of the meniscus

The Latin word *meniscus* comes from the Greek word *mēniskos* ''crescent'', diminutive of *mēnē* ''moon'' (Fox et al., 2012), the name is explicatory of the gross anatomy of this structure (Fig. 3).

Menisci are semilunar fibrocartilaginous structure interposed between the femoral and the tibial condyles: this characteristic shape is attained in humans between the 8th and 10th week of gestation [Gardner and O'Rahilly, 1968; Gray and Gardner, 1950]. Two menisci are present in each knee joint: a medial (*meniscus medialis*) and a lateral (*m. lateralis*) one [Evans and Lahunta, 2013]. The concavities of each meniscus look towards the centre of the joint where the two cruciate ligaments pass through. In cross section, the meniscus has a triangular shape with an inner and sharp-edged axial border (that looks to the intercondylar notch) and an outer and rounded-edged abaxial one (strictly in contact with the synovial capsule) [Messner and Gao, 1998; Evans and Lahunta, 2013] (Fig. 3, left side). Conventionally the meniscus is divided in an anterior or cranial horn, a central body and a posterior or caudal horn [Di Giancamillo et al. 2017], these structures are in continuity one with each other and this division should be considered more as a functional than as an anatomical partition (Fig. 3, right side).

Nevertheless, these differentiations have clinical and prognostic significance. Meniscal tears affected mostly the posterior region in human beings and in those animals that shows similar pathological patterns. On the other hand, tears of the inner zone show the worst therapeutic outcome [Mordecai et al., 2014]

The two horns allow the insertion of the meniscus to the proximal tibia throughout calcified or uncalcified fibrocartilaginous enthesis [Messner and Gao, 1998] whose sub-chondral insertions sites present specie-specific characteristic (See below, chapter 1.3).

Grossly, the human medial meniscus is more “C-shaped” and covers ˜60% of the medial articular surface; it is strictly attached to the proximal tibia and to the joint capsule, at the expense of its mobility during extension-flexion knee movements [as reviewed by Fox et al., 2012 and Fox et al., 2015].

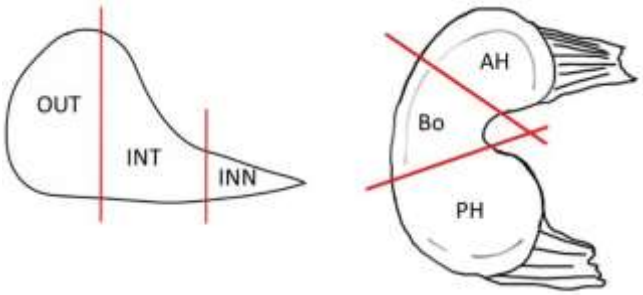

Fig. 3 Schematic representation of the different parts in which menisci may be divided: on the left, cross section of the meniscus; on the right, superior view. OUT: outer zone; INT: intermediate zone; INN: inner zone; AH: anterior horn; Bo: body; PH: posterior horn.

The lateral meniscus is more rounded than the medial one, it covers almost the 80% of the lateral articular surface and preserves its high mobility [as reviewed by Fox et al., 2012 and Fox et al., 2015]. The lateral meniscus displaced during knee flexion and extension twice as much than the medial one (see chapter 1.7) [Thompson et al., 1991].

The proportions of articular cartilage covered by the two menisci are relatively constant and they are similarly reported both in fetal and mature adult knee [as reviewed by Fox et al., 2015].

Specie-specific differences have been demonstrated even for this feature, for example, Brzezinski and colleagues (2017) reported that the percent coverage of the ovine medial meniscus (67.97%± 3.70%) was statistically greater than the coverage of the human medial meniscus (53.52 ± 3.13) [Brzezinski et al. 2017].

Dimensions of the human menisci vary depending on both the study considered and the applied measure method: Proffen et al. (2012) reported an anterior-posterior length of 39.8 mm and a width of 9.5 mm for the medial meniscus and an anterior-posterior length of ˜33 mm and a width of 9.8 mm for the lateral one [Proffen et al. 2012]; Takanori et al. (2016) reported an average width of ˜11.59 mm for lateral meniscus and of ˜10.55 mm for the medial one and a circumference of ˜101 mm and ˜103 mm respectively for the medial and the lateral menisci [Takroni et al 2016].

Differences in shape between medial and lateral menisci are similarly described even in dogs, where the lateral meniscus is slightly thicker and forms a slightly greater arc than the medial one. In large breeds the peripheral border of the lateral meniscus measures approximately 8 mm [Evans and Lahunta, 2013].

Even if the dimension of this structure is relatively preserved in the different human and animal species some structural differences in its morphology between human and animals (and among animals) are described and they may assume relevance in the process of selection of the best animal model for meniscus research [Proffen et al. 2012].

Proffen and colleagues (2012) evaluated these differences confronting gross anatomy of the menisci in human and in the principal knee animal models (cow, sheep, goat, dog, pig, and rabbit), focusing on both dimensions and insertion of the meniscus ligaments, and showed as the size and proportion of the human medial meniscus was most similar to the sheep and goat specimens while the lateral meniscus was most similar in size to the sheep, goat and pig specimens.

However, due to the anatomy of the tibial insertion sites of the lateral meniscus more similar to the human and considering both the medial and lateral meniscus, the suggested animal model was the goat [Proffen et al., 2012].

Oppositely, if focusing on the medial meniscus, the sheep seems to be the best animal model for human meniscus research [Proffen et al. 2012] due to the relatively well conserved anatomy of this structure between these two species: sheep knee may be seen as a scaled-down version of the human knee [Brzezinski et al., 2017] with only few, but noteworthy, differences: the anterior-posterior length, the circumference of the outer margin, and the meniscal volume are different between sheep and humans (Takroni et al., 2016) and the width of the posterior region is significantly greater compared with the anterior region in humans while the ovine meniscus is more symmetrical.

1.3. Ligaments of menisci

Each meniscus is strictly attached to the subchondral bone of the proximal epiphysis of the tibia throughout two, one for each horn, ligaments: the anterior (or cranial) and posterior (or caudal) menisco-tibial ligaments [Evans and Lahunta, 2013] (Fig. 4). These ligaments and their enthesis represent the anatomical continuation of the meniscal horns with the tibial plateau and the biomechanical function of the meniscus is strictly linked to the healthy state of these structure [Messner and Gao, 1998].

The attachment of these ligaments varies depending on the considered meniscus and present some degree of intra- and inter-specific variation. The anterior menisco-tibial ligament of the human medial meniscus present four different conformations. In the type I, the insertion is located in the flat anterior

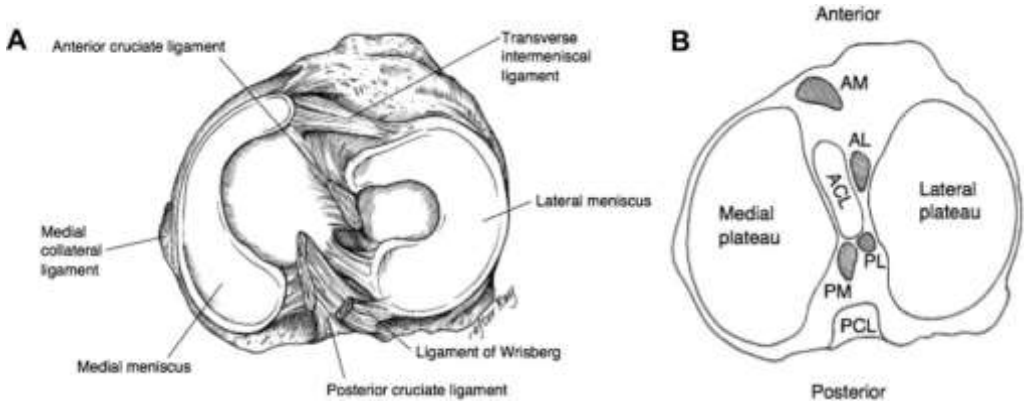

Fig. 4 A) Anatomy of the human meniscus viewed from above; (B) Axial view of a right tibial plateau showing sections of the meniscus and their relationship to the cruciate ligaments. AL, anterior horn lateral meniscus; AM, anterior horn medial meniscus; PCL, posterior cruciate ligament; PL, posterior horn lateral meniscus; PM, posterior horn medial meniscus. From Fox et al. 2015.

edge of the intercondylar region of the tibial plateau, just above the tibial tuberosity, this is the most common conformation among different species (human comprised) [Berlet and Fowler, 1998; Proffen et al., 2012].

The type II is characterized by the attachment in the downward slope from the medial articular plateau to the intercondylar region. And type III occurred on the anterior slope of the tibial plateau [Proffen et al., 2012].

Type IV presents no firm bony insertion of meniscal anterior horn [Proffen et al., 2012], it is described also in humans, but is largely expressed in canine species in which an intermeniscal ligament (*lig. transversum genus*), connects the anterior horn of the medial meniscus to the anterior horn of the lateral one [Proffen et al., 2012; Evans and Lahunta, 2013].

The attachment of the posterior menisco-tibial ligament of the medial meniscus occupies an area just anterior to the origin of the posterior cruciate ligament (PCL) on the lateral edge of the posterolateral surface of the medial spine in both human and animals' knees [Evans and Lahunta, 2013; Proffen et al., 2012 and as reviewed by Fox et al., 2012].

In humans, both horns of the lateral meniscus are located more centrally than the medial meniscus attachments, respect to other animals [Proffen et al., 2012].

The attachment of the human anterior menisco-tibial ligament of the lateral meniscus is on the lateral aspect of the lateral spine of the tibial intercondylar eminence [Proffen et al., 2012], just posterior and lateral to the anterior cruciate ligament (ACL) insertion [Fox et al., 2012]; while in cow, pig, sheep, goat and dog the attachment is close to the medial tibial spine [Proffen et al., 2012] (caudal to the transverse ligament in dogs [Evans and Lahunta, 2013]); and in the rabbit it is even more medial, very close to the anterior horn of the medial meniscus [Proffen et al., 2012].

The posterior horn ligament of the lateral meniscus of human inserts posterior to the lateral tibial spine and just anterior to the insertion of the posterior horn of the medial meniscus [as reviewed by Fox et al., 2012] and to the popliteal notch of the tibia, just caudal to the caudal intercondyloid area of the tibia, in dogs [Evans and Lahunta, 2013].

The medial meniscus does not present an independent menisco-femoral ligament, however its outer, thick and convex rim (aka *red zone*) is firmly interconnected with a condensation of the joint capsule that arise at the midpoint of the coronary ligament (the tibial portion of the capsular attachment), so much to form the so called *deep medial collateral ligament* [as reviewed by Fox et al., 2012].

In sheep the interconnection between the body of the medial meniscus and the medial collateral ligament is stronger than that of the human [Brzezinski et al., 2017]. On the other hand, lateral meniscus is loosely attached to the capsular ligament and its fibres do not attach to the lateral collateral ligaments as happens in the medial one. Lateral meniscus has peculiar attachments to the distal femur epiphysis named

menisco-femoral ligaments [Fox et al., 2012] (*lig. Meniscofemorale*) that represent the only femoral attachment for this meniscus [Evans and Lahunta, 2013].

Actually, there are discrepancies about the presence of these ligaments since it was described that it may be none, 1, 2 or 4 ligaments [Fox et al., 2012]. When present, these ligaments connect the posterior horn of the lateral meniscus to the lateral aspect of the medial femoral condyle crossing the joint space from lateral to medial and inserting close to the femoral origin of the posterior cruciate ligament [Fox et al., 2012], more inferiorly in human than in the animal specimens [Proffen et al., 2012].

Historically, if two menisco-femoral ligaments are present, the most anterior is named ligament of Humphrey while the most posterior is named ligament of Wrisberg [Fox et al., 2012].

The function of these ligaments may be to pull the posterior horn of the lateral meniscus in an anterior direction to increase the congruity of the menisco-tibial fossa and the lateral femoral condyle. [Fox et al., 2012].

Meniscal ligament entheses have a pivotal role in forces transmission during gait bearing and in the transformation of compressive forces into hoop stresses during joint loading [Messner and Gao, 1998]. Their nature was well reviewed by Messner and Gao (1998) in the leporine model. These Authors speculate that, since the anterior insertion of the menico-tibial ligament of the lateral meniscus showed a higher resistance to tensile failure test than the medial one [Goertzen et al. 1996] and seen that the latter was stronger than its posterior counterpart [Gao and Messner, 1996], the subchondral insertions of the lateral meniscus are subjected to higher tensile forces than those of the medial one, and that the anterior are subjected to higher loads than the posterior [Messner and Gao, 1998]. This hypothesis seemed to be confirmed by the described fine anatomical composition of these structures, that are: purely ligamentous in the anterior meniscal ligament, with longitudinally arranged collagen fibres and small fusiform cells aligned in rows, similar to the fibrocytes in ligaments; while, fibrocartilaginous in nature for the posterior one, with a lesser alignment of collagen fibres and the presence of more rounded cells.

These morphological differences have been ascribed to the different anatomical location and loading conditions of these two structures. In fact, in rabbits, the anterior insertional ligament is located in front of the joint away from any compressive forces and probably mainly loaded under tension, while the posterior one is placed more centrally within the area of contact between the femoral condyle and the tibial plateau and is probably loaded both under tension and compression.

1.4. Vascularisation of Menisci

At birth and during their initial development menisci are almost entirely vascular, with the blood supply entering from the periphery and extending through the entire width of the menisci [Clark and Ogden, 1983]. Blood vessels could be identified in the peripheral third of human foetus menisci around the 22nd

week of gestation [Petersen and Tillmann, 1995]. As the foetus continues to develop the vascular supply decrease and when complete maturity is achieved only the periphery of the structure presents vascular supply [Di Giancamillo et al., 2017]. This process of devascularization seems to be linked to the weight-bearing that occur since the first year of age in the human being: once an infant achieved a bipedal gait

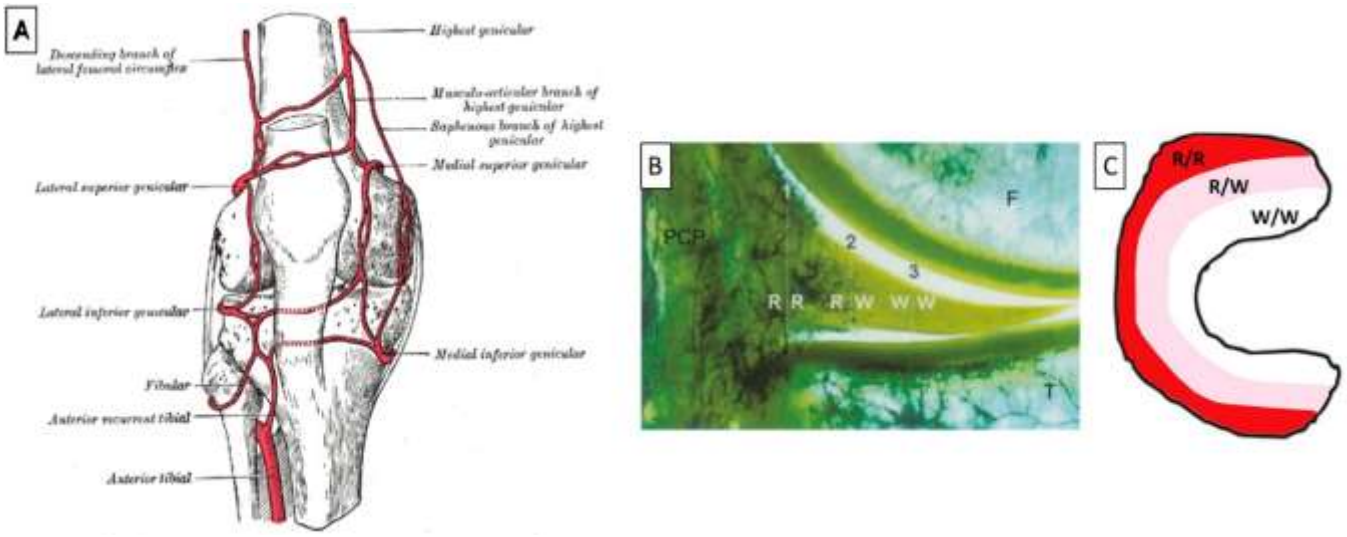

Fig. 5 A) Drawing of the main vasculature of the knee, from Gray, H. (1918); B) Frontal section of medial compartment. Branching radial vessels from the perimeniscal capillary plexus (PCP) can be observed penetrating the peripheral border of the medial meniscus. Three zones are seen: (1) RR, red-red area is fully vascularized; (2) RW, is at the border of the vascular area; and (3) WW, white—white is within the avascular area of the meniscus. F, femur; T, tibia; PCP, perimeniscal capillary plexus. R, red. From Arnoczky and Warren, 1982; C) Schematic drawing of the three meniscal zones seen from the above, drawn by UP.

pattern, the stress generates from body weight and muscular forces is too much for the blood vessels still present in the meniscal inner portion, and consequently this region becomes avascular [as reviewed by Gray, 1999]. In this way three different zones emerge: an outer, fully vascular area, called the *red-red zone*, strictly in contact with the joint capsule; an intermediate area, called the *red-white zone* (that present midway characteristic with the other two); and a completely avascular area, called *white-white zone*, that corresponds to the inner sharp edge of the meniscus [Di Giancamillo et al. 2017] (Fig. 5).

Vascular supply presents meniscus-specific features. The lateral meniscus is vascularized for its 10-25%, while the medial meniscus is more vascularized, with the 10-25% of its surface reached by the vascular network. The remaining portions of each meniscus (65% to 75%) receive sustenance from synovial fluid via diffusion or mechanical pumping provided by joint motion [as reviewed by Fox et al., 2012]. Even in this case, the weight-bearing seems to be essential for the healthy status of the meniscus since the diffusion of nutrients from the synovial fluid to the avascular portion requires a pump effect provided by an intermittent cycle of loading and releasing stress on the menisci via weight loading and muscular force [Gray, 1999]. The meniscus does not experience a significant amount of weight-bearing or muscular force during the first year of an infant's life and consequently the inner portion of the menisci cannot rely on diffusion from the synovial fluid and a direct blood supply to the entire meniscus is still necessary [Gray, 1999]. The amount of blood supply is directly correlated to the healing capacity of the meniscus [as

reviwed by Fox et al., 2012]. As the *Red-red zone* injuries are easier to repair than the lesions of the inner one (aka *white-white zone*) [Mordecai et al., 2014] for which the meniscectomy still remains the only suggested solution [Mordecai et al., 2014]. Meniscal injury in the young population have a better healing prognosis respect to those that happens to the adult or elder ones, due to the still present higher vascular supply [Vaquero and Forriol, 2016]. The vascularization of the menisci is provided by three branches of the popliteal artery: the medial, the lateral and the middle geniculate arteries [as reviewed by Fox et al., 2012] (Fig. 5A). These arteries form a perimeniscal plexus within the synovial and capsular tissues of the knee. [as reviewed by Gray, 1999]. These capillary plexuses enter in the meniscus via radial branches directed to the centre of the joint (Fig. 5B) [Arnoczky and Warren, 1982]. Endoligamentous vessels, from the anterior and posterior horns, form terminal loops into the substance of the menisci providing a direct route for nourishment [as reviewed by Fox et al., 2012]. The anterior and the posterior horns are more vascularized than meniscal body [as reviewed by Messner and Gao, 1998]. The entheses are vascularized as well, but not their fibrocartilaginous portions [Petersen and Tillmann, 1995].

1.5. Innervation of Menisci

The posterior branch of the posterior tibial nerve, the terminal branches of the obturator and femoral nerves and the peroneal nerve (for the lateral portion of the joint capsule) [as reviewed by Fox et al., 2012] are responsible for the innervation of the knee joint. Branches of these nerves (the posterior and medial articular nerves) provide the innervation to the menisci [as reviewed by Messner and Gao, 1998]. The fibres of these nerves penetrate the capsule and follow the previous described vascular path. The nerves fibres are amassed primarily in the peripheral portion of the meniscus [as reviewed by Fox et al., 2015] and mainly in the two horns [as reviewed by Fox et al., 2012; Messner and Gao, 1998; and Gray, 1999], while a poor or null innervation characterizes the body (the intermediate part is still innervated, even if lesser than the outer zone [as reviewed by Fox et al., 2012; and Gray, 1999]. The inner portion of the menisci, the *white-white zone,* is completely not innervated [as reviewed by Gray, 1999] (Fig. 6).

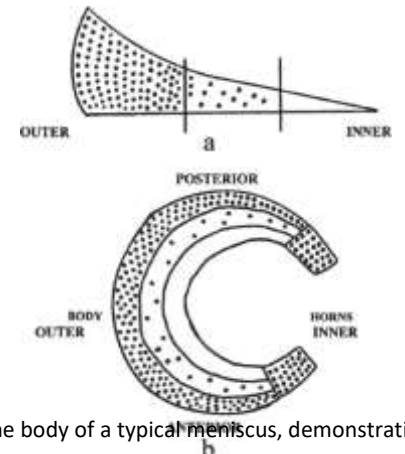

Fig. 6 a) Schematic cross-section through the body of a typical meniscus, demonstrating neural innervation. b) Schematic transverse section of a typical meniscus, demonstrating neural innervation of the horns and body. From Gray et al. 1999

Zimny et al. (1988) suggested that the neural innervation is mainly in the outer portion of the menisci because a need for joint and biomechanical realignment occurs only when compression is placed too far laterally on the menisci [Zimny et al., 1988] and a normal mechanics and pressure applied to the inner portion of the menisci would need no correction [as reviewed by Gray, 1999]. In addition, the posterior horns of both menisci have a higher concentration of nerves than the anterior horns [Zimny et al., 1988]. Different Authors [as reviewed by Gray, 1999] suggest that this may be due to the higher loads that focus on the posterior horn, associated to its less mobility (respect to the anterior horn) and to the higher pressure and tension derived from the compression carried from collateral ligaments when the knee is extended. Mechanoreceptors within the menisci act as transducers, converting the physical stimulus of compression and tension into a specific electrical impulse [as reviewed by Fox et al., 2012]. Different types of mechanoreceptors have been described in the menisci of both human and other animals (for examples, dogs and cats [Evans and Lahunta, 2013]). Type I (Ruffini endings) mechanoreceptors provide information to the central nervous system with information regarding static joint position, changes in intraarticular pressure, in direction amplitude, and velocity of joint motion [as reviewed by Gray, 1999; and Messner and Gao, 1998]. Type II (Pacinian corpuscles) mechanoreceptors are stimulate by tension changes, joint acceleration and deceleration [as reviewed by Gray, 1999; and Messner and Gao, 1998]. Type III (Golgi tendon organs) are activated by extreme motion or stress and provided protective reflex inhibition on the muscles operating on the knee joint [as reviewed by Gray, 1999; and Messner and Gao, 1998]. Moreover, free nerve endings (nociceptors) have been described in the horns and the outer two-thirds of the body of the menisci [as reviewed by Messner and Gao, 1998]. Free nerve endings have been described even in the meniscofemoral ligaments of Humphry and Wrisberg, in the meniscal transverse ligament [Biedert et al., 1992] and in the entheses' uncalcified and calcified fibrocartilages [as reviewed by Messner and Gao, 1998]. As previously described for meniscal capillaries, the number of nerve endings decrease with growth [Assimakopoulos et al., 1989].

1.6. Ultrastructure and Biochemistry of Meniscus

Menisci are fibrocartilaginous structures; thus, they show midway characteristics between fibrous tissues (i.e. tendons) and articular cartilage.

The different phenotypes of cells that are present within the meniscus mirror such similarities.

They are distributed in a regional-specific manner (Fig. 7). In the outer zone, fibrocyte-like cells (oval/fusiform in shape and with long cell extensions) reflect the fibrous pattern of meniscus while, in the inner zone, chondrocyte-like cells (more rounded) are responsible of the cartilaginous aspect of this zone. Furthermore, a third type of cells are present in the most superficial zone, these cells are flat and with no cellular extensions and seems to act as specific progenitor cells with therapeutic and regenerative properties [as reviewed by Makris et al., 2011; and Vaquero and Forriol, 2016].

These regional variations are acquired during the latter stage of development, since initially menisci show no phenotypical differentiation [as reviewed by Makris et al., 2011].

- **Meniscal cells:**

Meniscal cells are embedded in a specialized Extracellular Matrix (ECM) whose composition may varies due to physiological (i.e. during growth/aging) and pathological conditions (i.e. meniscal injuries/degeneration).

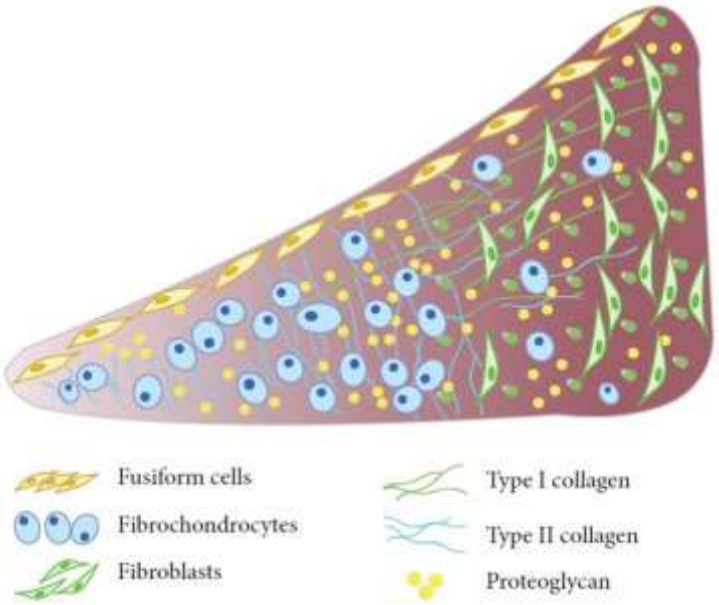

Fig. 7 Schematic diagram of meniscus internal ultrastructure. Most of collagen fibers bundles oriented circumferentially; only few of them presented a radial alignment. Cells of the superficial zone are fusiform in shape and located below the tissue surface; the cells in the outer one-third region or vascular zone are mainly elongated fibroblasts, and collagen I accounts for >90%; for inner two-thirds region or avascular zone, round or oval shaped fibro-chondrocytes are interspersed, the ratio of collagen I and collagen II is about 2:3. Partially modified from Niu et al., 2016.

The physiologic ECM composition of a mature human meniscus is mainly composed of collagen fibres (75% of the organic matter composing the meniscus), Glycosaminoglycans (GAGs, 17% of the organic matter composing the meniscus), adhesion glycoproteins (<1%) and elastin (<1%) [as reviewed by Makris et al., 2011].

- **Collagen fibres:**

Collagen fibres are mainly of type I, 98% of the total collagen content [McDevitt and Webber, 1990], with some other variants (II, III, IV, VI, XVIII) which represent less than the 1%. However, regional specialization involves even the distribution of the different types of collagens, with a predominance of collagen type I all over the red-red zone and the co-presence of collagen type I and II (40:60) in the white-white zone [Cheung, 1987]. Same distribution has been described in canine and ovine menisci [Kambic and McDevitt, 2005; Melrose et al. 2005].

Type VI collagen is also abundant in meniscus, constituting approximately 1–2% of tissue dry mass [Wu et al., 1987], and has been described in the interterritorial and pericellular / cellular compartments of the ECM [as reviewed by Vanderploeg et al., 2012].

The arrangement of structural collagen fibrils follows two main configurations (Fig. 8).

Circumferentially oriented collagen fibres, typically of collagen type I, pass through the body of the meniscus from a horn to the other and continues in the anterior/cranial and posterior/caudal menisco-tibial ligaments; this arrangement is the predominant within the meniscus [Skaggs et al., 1994].

Perpendicular to the latter, some fibres are arranged radially (i.e. from the outer to the inner zone of the meniscus). These fibres are paired laterally to form sheets of collagen that bifurcate and come together to form a honeycomb-like network which entrapped the circumferential bundles [Skaggs et al., 1994; Rattner et al., 2011] in a unique construct that act as a scaffold for the meniscus.

Weight-bearing and joint motion during development are implied in determining the orientation of the collagen fibers [Fox et al. 2015].

Regional differences have been described for the fibres arrangement and it has been supposed that this organization has a direct relationship to the load-bearing function of the menisci [Hellio le Graverand et al., 2001 a and b; Chevrier et al., 2009; and Rattner et al., 2011] (see chapter 1.7).

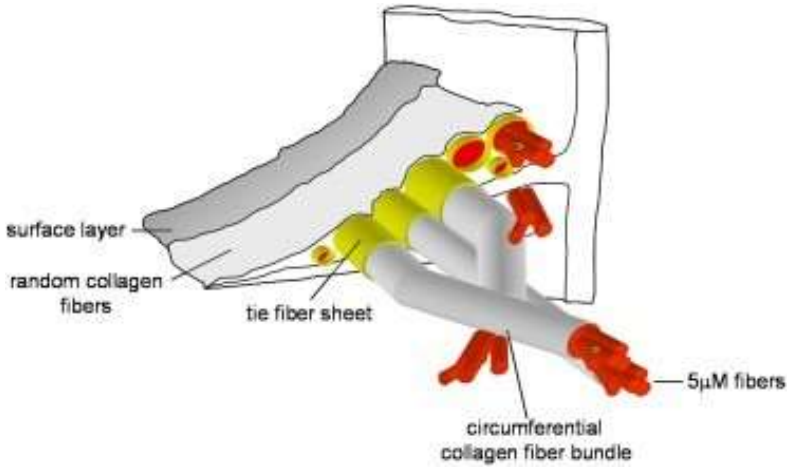

Fig. 8 Diagrammatic representation of the complex model of the meniscus. Underneath the surface layers of collagen fibrils and cells there are circumferential collagen fibril arrays (red) that are enveloped by a honeycomb-like network of radial tie fibril sheets (yellow). From Rattner et al., 2011

The most superficial collagen bundles are randomly orientated with a composition that resembles to articular hyaline cartilage [Beaupre et al., 1986; McDermott et al., 2008], this arrangement allows low-friction movement during knee motion, when the femoral condyles slide against the menisci and move it over the tibial plateau.

Radial fibres are mainly constitute of collagen type II and are observed mainly in the inner zone of the meniscus [Kambic and McDevitt, 2005] and in its femoral surface [Peretti et al., 2019], less-frequently, radially-orientated collagen fibre can also be found within the bulk of the meniscal tissue and may act as

tie fibres [as reviewed by Vanderploeg et al., 2012] these bundles are constitute of both type I collagen and II [Kambic and McDevitt, 2005].

When compression is applied upon meniscus, these fibres, that envelope the longitudinal bundles, avoid their separation on the transverse plane [as reviewed by McDermott et al., 2008].

Moreover, elastin fibrils are aligned at intervals along the radial collagen fibrils [Rattner et al., 2011] implying for a mechanical role of this type of fibres in the elastic properties of the meniscus.

On the other hand, seen their relationship with menisco-tibial ligaments, circumferential fibres, constituted of collagen type I, represent the structural component that allows the menisci to resist tensile hoop stresses in the circumferential direction [Rattner et al., 2011]. These fibres are described mainly in the middle and outer part of the meniscus [Kambic and McDevitt, 2005] and in its tibial surface [Peretti et al. 2019].

These are the reasons because lesions that affect the radial fibres are prognostically worse than those that affect the longitudinal ones: a radial transection through the meniscus or its insertional ligaments will completely disable the load distribution function of the meniscus [Messner and Gao, 1998]. In contrast, the menisci still transmitted significant proportions of the load in vitro after excision of a central segment of the meniscal body when the peripheral circumferential fibres and insertional ligaments were left intact [Burke et al., 1978].

Meniscal cells are associated with collagen bundles suggesting that they are integrated within the matrix elements and function as a unique ‘‘unit’’ [Rattner et al., 2011].

- **ECM - proteoglycans**

Further functional components of meniscal ECM, with a clear role in the biomechanics of the meniscus, are the proteoglycans (PGs). Proteoglycans are large, negatively charged hydrophilic molecules formed by a core protein with one or more covalently bound glycosaminoglycan (GAGs) chains. The size of PG may be increased by specific interaction with hyaluronic acid (as reviewed by Fox et al., 2012).

The concentration of proteoglycans across the width tissue is variable: the inner surface of the meniscus [Mahmood et al., 2019; and as reviewed by Vanderploeg et al., 2012] and the horns [as reviewed by Fox et al., 2012], which are exposed to the highest compressive loads, presents the higher concentrations of these molecules [Nakano et al., 1997]. In adult porcine menisci, glycosaminoglycans constitute approximately 8% of the dry mass of the inner region but only 2% in the outer region [Nakano et al., 1997]. Proteoglycans are responsible for hydration and provide the tissue with a high capacity to withstand compressive loads [as reviewed by Fox et al., 2012 and 2015]].

These large proteins, immobilised within the collagen network [Furumatsu et al., 2011] in a low permeable structure, generate an osmotic pressure gradient that allow fluid distribution: water molecules are expelled from the tissue (under load) and are re-captured (during unloading), due to their attraction for

the negative charge of the PGs, until the osmotic gradient within the meniscus does not return to its equilibrium [Mahmood et al., 2019].

This mechanism is called “ionic effect” and approximately 79% of the stiffness of the meniscus seems to be ascribed to it [Mahmood et al., 2019]. The depletion of GAGs causes a significant decrease in the elastic modulus and an increase of the permeability of the meniscus [Abdelgaied et al., 2015].

Moreover, the flow of the water fraction in and out of the meniscus allows the exchange of nutrients between the synovial fluid and meniscus [Fithian et al., 1990].

Different types of proteoglycans are present throughout the meniscus: aggrecan, perlecan, decorin and biglycan, are the main described.

Aggrecan is the major PG in human and animal menisci and is thought to be largely responsible for their viscoelastic compressive properties. It is composed, as the others PG, by a core protein to which are covalently attached different GAG chains [as reviewed by Fox et al., 2012]. Aggrecan does not exist in isolation within the extracellular matrix but only in the form of aggregates [as reviewed by Fox et al., 2012] composed of a central filament of hyaluronan (HA) with multiple aggrecan molecules bonded to it non-covalently via one terminus of their core proteins (Fig. 9). The GAGs furnish high negative charge to aggrecan, whereas aggregation gives it a large size. Both the charge and size properties are essential for the aggrecan’s functions [as reviewed by Fox et al., 2012]. Aggrecan has a complex interaction with collagen type II fibrils. The type II collagen and aggrecan in the tie fibers and in the fibrils that surround the bundles of tensile-bearing circumferential fibril, may provide a compressive cushion when meniscus is under load and the circumferential fibrils are under tension (Valiyaveettil et al., 2005).

Smaller proteoglycans, such as decorin, biglycan, and perlacan, are also sporadically found [as reviewed by Fox et al., 2012].

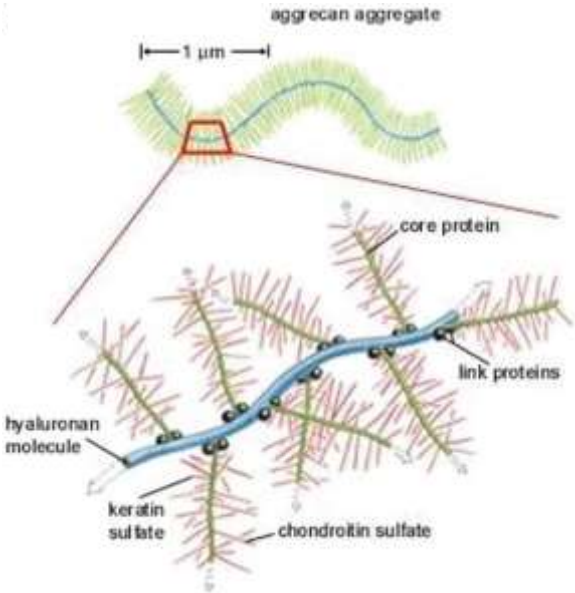

Fig. 9 Extracellular matrix. Schematic drawing of an aggrecan aggregate. From Fox et al. 2012

Perlacan is a heparan sulphate (HS) or chondroitin sulphate (CS) substituted proteoglycan whose name derives from the pearls-on-a-string-like appearance that it displays in rotary-shadowing experiments exhibiting an 80 nm long core protein and 5–7 variably sized globular domains [Melrose et al., 2008]. Its localization is principally pericellular, mainly associated to the rounded chondrocytic-like cells of the inner zone and to the distribution of collagen type II fibres [Melrose et al. 2005]. Thus, it is recognized as a marker of the early chondrocytic phenotype acquisition [Melrose et al. 2005].

Perlacan enhances collagen fibril formation, principally orientating appropriately type XI collagen molecules on the surface of type I and II collagen fibrils to direct fibrillogenesis. [as reviewed by Melrose et al., 2008], Moreover, it is involved in the formation of the cell–cell and cell–ECM interconnections with an important role in mechanosensory processes [as reviewed by Melrose et al., 2008].

Decorin, so named for its ability to bind to and "decorate" collagen fibrils, binds to and regulates collagen fibrillogenesis acting on the regulation of collagen fibrils diameter and fibrils orientation to achieve the proper assembly of its network [as reviewed by Melrose et al., 2008].

Biglycan and decorin exhibit opposite distributions in the adult rabbit menisci, with biglycan being more prevalent in the inner regions and decorin more prevalent in the peripheral regions [as reviewed by Vanderploeg et al., 2012].

Decorin interacts with multiple collagens to create functional bridges between the pericellular matrix and the surrounding extracellular matrix and, in association with collagen type VI, is essential in cellular resistance to deformation [Twomey et al., 2014].

The distribution of one PGs or another may be modified by endogenous factors, such as aging, or external factors, such as biomechanical forces. Minimal biomechanical disturbances acting upon cells within ECMs can modulate cellular phenotypic expression and modify the ECM composition.

1.7. Biomechanics and Biokinetics

The behaviour of the menisci in reaction to applied loads results from their macro-structure, their micro-composition and from the nature of their insertional ligaments [McDermott et al. 2008; Fox et al., 2015]. Due to their insertions on the tibia, menisci are not completely free to move within the knee joint.

So, the result of the application of a compressive force upon these structures, such that applied by the femoral condyles during load bearing, is the generation of a tension that is transferred within the meniscal tissue, along the circumferential collagen fibres, to the meniscal insertions. These tensile force stretches the circumferential wavy fibres and consequently, the circumference of the meniscus increase generating a circumferential tension within the meniscus [McDermott et al. 2008]. As a tissue is stretched, a force

will develop within the tissue, opposing to its elongation [McDermott et al. 2008]. This mechanism, that is known as *hoop stress*, avoids further meniscal deformation [Jones et al., 1996] (Fig. 9).

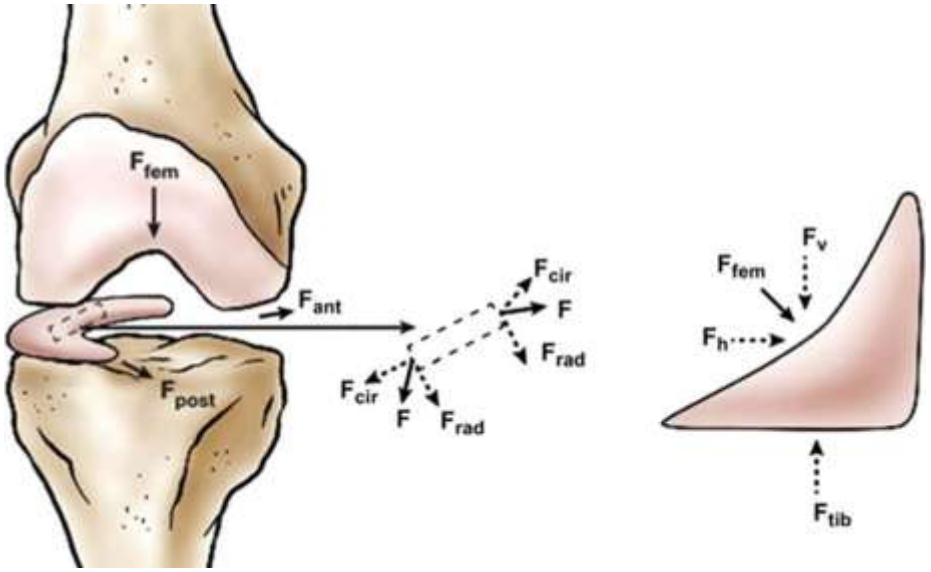

Fig. 10 Free body diagram of forces acting on the knee human meniscus during loading. During normal loading, the meniscus is compressed by the downward force of the femur. The meniscus deforms radially but is anchored by its anterior and posterior horns (F_{ant} and F_{post}). During loading, tensile, compressive, and shear forces are generated. A tensile hoop stress (F_{cir}) results from radial deformation, while vertical and horizontal forces (F_v and F_h) result from the femur compressing the curved superior surface of the tissue. A radial reaction force (F_{rad}) balances the femoral horizontal force (F_h). From Makris et al., 2011.

Moreover, meniscus, since it is composed of a solid matrix (consisting predominantly of the circumferentially orientated collagen bundles) and an interstitial fluid phase (mainly composed by water), acts as a biphasic material [Favenesi et al., in 1983].

The liquid phase of the meniscus, being free to move in and out the network of the solid phase, can be exchanged with the surrounding fluid [McDermott et al., 2008]. These may be consequence either of a mechanical compression or the direct application of a pressure differential (such as the oncotic pressure generated by PGs). The fluid component of the tissue is extruded at a rate dependent on the permeability of the tissue and the viscosity of the fluid [McDermott et al., 2008].

Thus, simultaneously to the application of a load to meniscal tissue, the solid phase shows an elastic response, as seen previously, while, on the other hand, the liquid phase and the permeability of the meniscal tissue are responsible for its viscoelastic response that allow to the this component to carries a significant part of the applied load [Spilker et al., 1992].

It has been showed that the lateral meniscus carries 70% of the load of the in lateral compartment, and that the medial meniscus carries 50% of the load in the medial compartment [Seedhom, 1976].

Both the liquid and the solid phases gives to the meniscus its capacity to absorb shocks: during loading of the joint, axially directed energy across the knee is converted into hoop stresses within the circumferential collagen fibres [Krause et al., 1976], and, as well as energy is absorbed into the collagen fibres (solid phase

of the meniscus), it is further absorbed by the expulsion of the joint fluid (fluid phase of the meniscus) out of the tissue.

The shock absorbing capacity of the menisci has been measured and it has been shown that without the menisci shock absorption within the knee is reduced by approximately 20%. [Voloshin and Wosk, 1983].

The important role of the meniscus in load transmission and shock absorption is well known [as reviewed by Sandmann et al., 2013]: it has been demonstrated that approximately 50–70% [as reviewed by Vanderploeg et al 2012] of load acting on the extended knee joint is transmitted to the meniscus (65–70% lateral and 40–50% medial) [as reviewed by Fox et al., 2015]. In flexion, this value increases up to 90% [as reviewed by Fox et al. 2015]. Consequently, loss of meniscus tissue leads to focal overload of articular cartilage and finally to a higher incidence of osteoarthritis [as reviewed by Sandmann et al., 2013] (Fig. 11).

After total meniscectomy, joint contact areas decreased by approximately 75%, and peak local contact stresses increase (as reviewed by Fox et al., 2015], by approximately 235%. [McDermott et al., 2008].

Knee joint possess a six degree of freedom movements [Li et al. 1996]:

1. flexion-extension,
2. abduction-adduction,
3. external-internal rotation,
4. lateral-medial translation,
5. anterior-posterior translation, and
6. superior-inferior translation.

In a so-mobile joint, to maintain the congruency between two very incongruent surfaces, such as the spherical-like femoral and flatten tibial condyles, menisci have to act as dynamic structures that follow the movements of femur and tibia [McDermott et al. 2008].

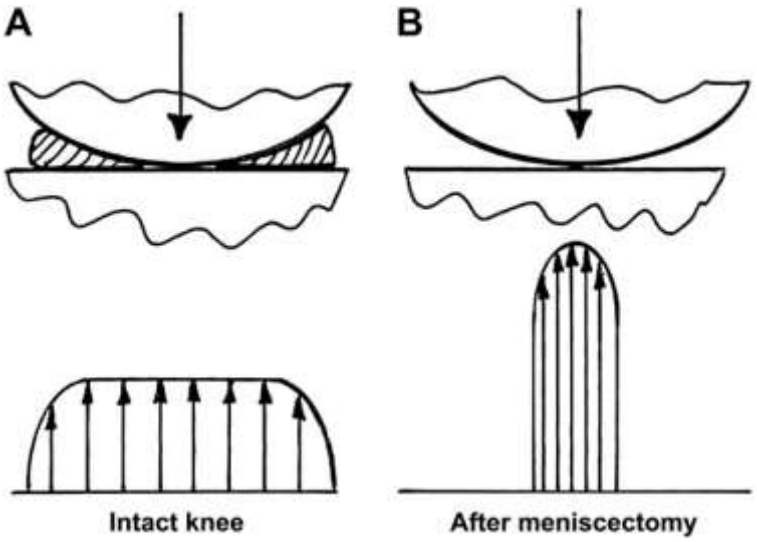

Fig. 11 Local contact pressures before (A) and after (B) meniscectomy. From McDermott et al., 2008

Vedi and colleagues (1999) have described in details meniscal movements in the normal human knee, during both weight-bearing and non-weight-bearing situations [Vedi et al. 1999] (Fig. 12). These Authors observed that both the menisci move posteriorly during knee flexion whit the lateral meniscus that is more mobile than the medial one [Vedi et al. 1999; Fox et al., 2015]. Other Authors quantify this posterior displacement of the lateral meniscus as twice as much that of the medial one [as reviewed by Messner and Gao, 1998].

Moreover, the anterior horns show a higher mobility than the posterior one [Vedi et al., 1999].

All these findings make the posterior horn of the medial meniscus the less mobile part [Vedi et al., 1999].

When the values observed in the unloaded knee are compared with those measured in the loaded one, a significant greater movement in the anterior horn of the lateral meniscus has been described in the latter situation, however, no significant differences are noted in the other meniscal movements [Vedi et al., 1999].

Moreover, as the femoral condyles bear down upon the menisci, they tend to be extruded peripherally [Krause et al., 1976] and this is the first step for the hoop stress mechanism activation.

In dogs, where the tibial plateau possesses a characteristic high convexity and is more posteriorly sloped than in humans, during flexion, menisci move significantly in both the cranial/caudal and superior/inferior directions [as reviewed by Park et al., 2018].

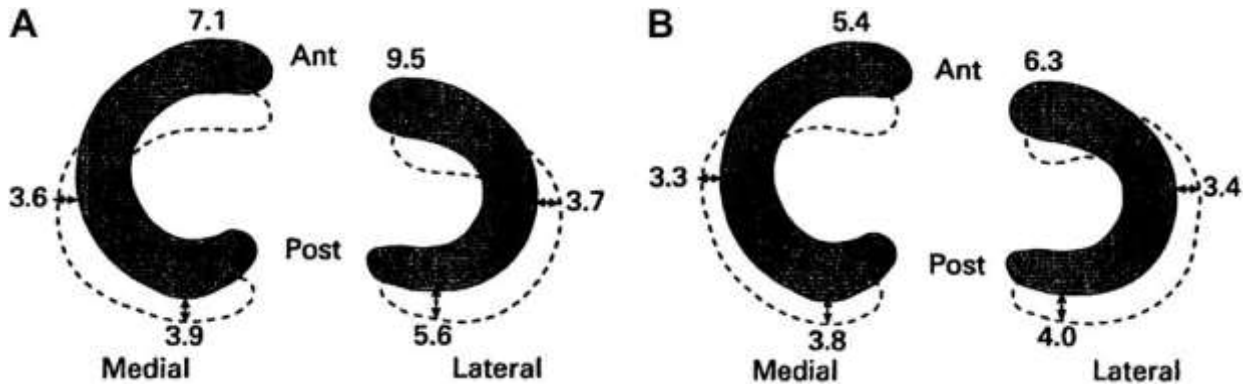

Fig. 12 Diagram showing the mean movement (mm) in each meniscus from extension (shaded) to flexion (hashed) in (A) the weight-bearing and (B) the unloaded knee. From Vedi et al., 1999

1.8. Development, maturation and aging

- **Development**

Menisci arise from a condensation of cells in the intermediate layer of the mesenchymal tissue [as reviewed by Fox et al., 2015].

The origin of these cells is explicatory of the dichotomic nature of menisci, as it has been described in mice how these cells derive from two different strains of the joint embryonic gem: the cells that compose the

inner zone of the medial meniscus derives from resident cells of the limb bud that switch off expression of collagen type II to form the interzone at day 13.5 of embryo's development; on the other hand, the lateral meniscus and the outer medial meniscus derive from cells that invade the developing knee joint from the area adjacent to the interzone at day 14.5 [Hyde et al. 2008].

Different studies focused on meniscal formation and development were performed in murine and chicken models and in human fetus. These studies reported that meniscus formation happens at 8 days in chicken embryos [Mikic et al., 2000], at 15 days in mice embryos [Gamer et al. 2017] and as early as the 7^{th} weeks of gestation in human embryos [Uhthoff and Kumagai, 1992]. While, the characteristic shape of menisci is attained in humans between the 8th and 10th week of gestation [as reviewed by Fox et al., 2012].

Neonatal meniscus shows several differences respect to the mature one.

The developing menisci are highly cellular and fully vascularized [as reviewed by Fox et al., 2012]. Blood vessels could be identified in the peripheral third of human foetus menisci around the 10^{th} week of gestation and subsequentially invade entirely the tissue [Uhthoff and Kumagai, 1992]. However, during meniscal development, both cellularity and vascular supply continue to decrease, while, GAGs increase in the inner zone, and collagen fibres pass from a disorganized arrangement to the previously described one [as reviewed by Fox et al., 2012]. Nevertheless, the collagen fibres present in the early stages of meniscal maturation are composed only of collagen type I, type II collagen will be expressed only in later stages of development and primarily in the inner zone [Melrose et al., 2005; Di Giancamillo et al. 2014].

Joint motion and the postnatal stress of weight-bearing are the principal factors that determine the phenotypical and architectural changes that characterize the maturation process of the meniscus.

Mikic et al. (2000) described the enormous dependence of this structure on biomechanical force application for its correct development: these Authors observed the degeneration and the consequent disappearing of meniscus during its formation in only two days after joint immobilization [Mikic et al., 2000].

Biomechanical stimuli modify the environment within and around menisci, principally causing devascularization of this structure [as reviewed by Gray, 1999]. It has been described how the different factors that regulate vascularization and those that regulate the biochemical composition are regulated by a common pathway activates by mechanical stimuli [Di Giancamillo et al., 2017]. Angiogenesis is regulated by a balance between stimulatory and inhibitory molecules [as reviewed by Di Giancamillo et al., 2017].

Among the pro- angiogenic factors, VEGF is one of the most important [as reviewed by Di Giancamillo et al. 2017], and it has been associated to the healing of meniscal injuries [as reviewed by Di Giancamillo et al. 2017], nonetheless, its action may depend on the considered region of the meniscus, since it seems to have no effects on the inner and physiologically avascular region [Di Giancamillo et al. 2017]. On the contrary, endostatin is the principal exponent of the antiangiogenic factors and seems to have a role in

counteracting the VEGF-induced effects. Both the expression of endostatin and VEGF are influenced by mechanical factors [as reviewed by Di Giancamillo et al. 2017].

VEGF expression decreases from neonatal to adult animals, associated to the decrease of vascularization that characterize this transition [Di Giancamillo et al. 2017]. Endostatin, on the other hand, seems to have a role in matrix development and maintenance of meniscal avascular zones [as reviewed by Di Giancamillo et al. 2017]. The major expression of endostatin has been described in the young subjects which are characterised by an increasing in joint motion and load-bearing [Di Giancamillo et al. 2017] and consequently to a switch between the expression of SOX-9 (considered as an early marker of chondrogenesis), that decreases, and Collagen II (whose expression is considered the fulfilment of meniscus maturation), that increases [Di Giancamillo et al. 2014].

- **Maturation**

Maturation of menisci is achieved when both cells and ECM get their functional role in the tissue. That means a complete differentiated phenotyping for the former and the achievement of the bio-mechanical competence for the latter.

The previously described phenotypical cells regional variations (see chapter 1.6) are acquired during the latter stage of development, since initially menisci show no phenotypical differentiation [Makris et al., 2011]. Thus, also the expression of the different proteins of the ECM varies during growth [Melrose et al., 2005; Vanderpoleg et al., 2012; Di Giancamillo et al., 2014].

In the early stages of development, aggrecan and perlacan are present in the inner zone, confirming the cartilaginous-like nature of this region, then in the later stages aggrecan is spread all over the tissue with the regional variation previously described (see chapter 1.6); in contrast, perlecan, being strongly associated to the pericellular matrix, tends to decrease with the onset of age and the consequent decellularization process [Melrose et al., 2005].

Even decorin and biglycan showed an opposite behaviour, with decorin that acts as the dominant, endogenous, non-aggregating proteoglycan in the adult meniscus and cartilage [Sampaio et al., 1988; Roughley and White, 1992] while, in immature articular cartilage, and so probably also in meniscus, biglycan is present at much higher concentrations than decorin [Roughley and White, 1989].

Meniscal maturation is the consequences of a balanced relationship between biomechanical forces and the adaptive modification undertaken by the tissue.

- **Aging**

The natural process of meniscal senescence is characterized by the loss of this balance and [as reviewed by Krupkova et al., 2018] includes, besides the decreased cell function and density, the loss of the arrangement of collagen fibers and the dehydration of the tissue [Krupkova et al., 2018].

These aging-related modifications are associated to detrimental changes in meniscal material properties [as reviewed by Krupkova et al., 2018] (see chapter 1.7) that generate anisotropies in the ECM and lead to variations in the distribution of local stress and strain and consequently alter cells and ECM responses to mechanical loading [as reviewed by Krupkova et al., 2018].

The structural disorganization of the ECM, associated to the reduced meniscal repair capacity due to devascularization of the white-white zone, can progress to meniscal lesions [as reviewed by Krupkova et al., 2018] even after physiological loading [as reviewed by Krupkova et al., 2018].

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2. AIM

This thesis was thought with the intent to try to fill some lacunae in the knowledge of the anatomic-dependent features of the meniscus.

In particular, the study of the effects of endogenous and exogenous factors upon the development of this structure will be outlined.

- Endogenous factors have been considered those factors that cannot be attributed to external or experimental factors: the effect of age and growth has been investigated focusing on the variation of the, matrix components (collagen types, GAGs and decorin; Chapter 3.1 and 3.2), cellular phenotypic modifications (Chapter 3.1 and 3.3) and meniscal morpho-functional structure (Chapter 3.3), with additional focus on possible differences presented in different animal models.
    - 3.1 Meniscus matrix structural evaluation: immunohistochemical, biochemical and biomechanical age-dependent properties in a swine model.
    - 3.2 Decorin age-related variations in the distribution of pig extracellular matrix meniscus
    - 3.3 Postnatal morpho-functional development of dog's meniscus.

On the other hand, exogenous factors have been considered those factors that are in any way attributable to the external interventions operated by the experimenter or by the application of forces upon meniscus.

- Exogenous factors: the effects of physiologic (compression and traction to which meniscus is naturally subjected; Chapter 4.1) and non-physiologic (continuous compression without flexion; Chapter 4.2) forces applied to meniscus during growth were evaluated in this section. Furthermore, the effect of hypoxia (Chapter 4.3) in meniscal tissue-culture was also evaluated as exogenous factor in a neonatal committed cell population in order to assess a faster maturation of the tissue.
    - Biomechanics-related physiological differentiation:
        - 4.1 Swine meniscus: are femoral-tibial surfaces properly tuned to bear the forces exerted on the tissue?
    - Compression force effect upon meniscal matrix
        - 4.2 Effects of continue compressive forces upon meniscal matrix in a population of 12 Dobermann Pinschers affected by quadriceps contracture syndrome.
    - Environmental hypoxia upon meniscal cells
        - 4.2 Hypoxia as a stimulus upon neonatal swine meniscal cells: highway to phenotypic maturation of the meniscal chondrocytes?

The importance of these investigations is linked to the possible application of these notions in the field of tissue engineering of the meniscus and may improve the current knowledge on the morpho-functional effect that external factors exercise on its structure.

3. Endogenous Factors

3.1. MENISCUS MATRIX STRUCTURAL EVALUATION: IMMUNOHISTOCHEMICAL, BIOCHEMICAL AND BIOMECHANICAL AGE-DEPENDENT PROPERTIES IN A SWINE MODEL

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Abstract

Composition, structure and biomechanics are basic fundamental information for engineering meniscal substitutes. The analysis of the morphological, structural, biochemical, and mechanical changes, which occur during growth of the normal meniscus, represent the goal of the present study. Menisci from adult pigs (9-month old), young (1-month old), and neonates (stillbirths) were collected. Cellularity and glycosamiglycans (GAGs) deposition were evaluated, while Collagen 1 and aggrecan were investigated by immunohistochemistry and Western blot analyses in order to be correlated with biomechanical properties of traction and compression tensile forces, respectively. Cellularity decreased from neonatal to adult and GAGs showed the opposite trend ($P<0.01$ both). Collagen 1 decreased from neonatal to adult, as well as the ability to resist to tensile traction forces ($P<0.01$), while aggrecan showed the opposite trend, concordant to biomechanics: compression test showed that adult meniscus greatly resists to deformation ($P<0.01$). This study demonstrated that in swine meniscus clear changes follow the meniscal maturation during growth: starting with an immature cellular and fiber pattern (neonatal) to the mature organised and differentiated meniscus of the adult.

Keywords: Meniscus, pig, anatomy, matrix, biomechanics, development

1. Introduction

The menisci are fibrocartilaginous structures interposed between femoral condyles and tibial plateau of all mammals. They are fundamental for the knee balance, because the femoro-patellar-tibial joint is, basically, an imperfect hinge [Proffen et al., 2012]. Furthermore, the knee kinematics is characterized by a three-plans movement, that is, different moduli of solicitation develop in this joint [Yoshioka et al., 2007]. Therefore, menisci have multiple functions in this articulation: they act as shock absorbes, they can bear loads and allow joint stability, congruity and lubrication [Fox et al., 2012]. If the stifle presents a structural abnormality, or a previous or concomitant disease, the different load and stress distribution over the two menisci will lead to meniscal injuries and developing of osteoarthritis (OA) due to the failure of meniscal protective function in the joint [Howell et al., 2014]. For these reasons, the recognition of the clinical importance of the meniscus has led the development of more detailed studies regarding this particular anatomical structure [Di Giancamillo et al. 2016, Peretti et al. 2017].

The meniscus is an inhomogeneous and anisotropic material, because its proprieties vary non-linearly with location and direction of stimuli, depending on distribution and organization of its components: water, collagen and GAGs (principally aggrecan), which co-work to resist to the different forces that act on menisci. Collagen type I is arranged in circumferential bundles all along the inner part of meniscus and, co-expressed with collagen type II, in radial bundles [Abraham et al., 2011]; this disposition of the fibres reflect the directions of the two principal forces that act on meniscus: circumferential/tensile force and gait/load force. Aggrecan has a complex interaction with collagen type II fibrils. Thereby, aggrecans enable the tissue to resist compressive forces: by locking itself within the collagen meshwork and absorbing large amounts of water from the surrounded environment, exert a swelling pressure on the collagen meshwork [Valiyaveettil et al., 2005]. Within the extracellular matrix (ECM), collagen fibrils resist to such swelling. If aggrecan and collagen are in equilibrium, the tensile forces that stretch the collagen fibrils balance the swelling of the aggrecan. During compression, this equilibrium is perturbed: compression displaces water and this increases the swelling potential of the aggrecan. Upon removal of the compression, the aggrecan will re-swell and restore the original equilibrium [Roughley and Mort, 2014]. Therefore, the complex disposition of collagen fibrils in the meniscus supplies its tensile properties, whereas aggrecan expresses important weight-bearing properties in this fibro-cartilaginous tissue [Melrose et al., 2005].

Furthermore, a stress over meniscal tissue leads to a different response by fibrocartilage that is an intrinsic viscoelastic and an extrinsic poroelastic response. More precisely, under normal physiologic loading, the meniscus experiences high tensile and shear stresses as well as compressive stress [Shrive et al., 1978], which can lead to injury. Unfortunately, the meniscus is characterized by the lack of vascularisation [Arnoczky and Warren, 1983; Arnoczky and McDevitt, 2013; Pufe et al., 2004; Smith, et al., 2010; Di Giancamillo et al., 2017], which is confined in its outer third in the adult meniscus, so, its regeneration and ability to repair are extremely scarce, if not absent, especially in the avascular zone.

Many therapeutic procedures for meniscal tears have been applied over the years [Deponti et al., 2015]. None of these procedures, however, has the capacity of preventing the development of osteoarthritis in the knee joint. Nowadays, the treatment goal is not just to remove the damaged part of the meniscus, but rebuild it or, eventually, replace it. Of course, this idea is still far from its immediate realization, although many attempts have already been made [Yu et al., 2015; Ding and Huang, 2015; Wei L-C et al., 2012]. For this reason, more studies are necessary to increase our knowledge about this small but essential structure. Meniscal composition, structure and biomechanics are basic fundamental information for engineering meniscal tissue substitutes. The goal of the present study is represented by the characterization of the morphological, structural, biochemical, and mechanical properties of the normal meniscus and their changes during growth.

2. Materials and methods

For the aim of this study we obtained menisci from a local slaughterhouse, which breeds swine Landrace x Large White. Knees of adults (9-month old, weight 100 kg), young (1-month old, weight 10-12 kg), and neonates (stillbirths or dead in peripartum) were collected (Fig. 1A).

The joints were dissected to isolate lateral and medial menisci. Capsular tissue and ligaments were gently removed, and the menisci were stored in saline solution NaCl 0.9% and refrigerated. No animal has been sacrificed for the purposes of this study. All animals that were used were died for reasons that have no relationship with the present study.

2.1 Biochemical analyses.

Giving the similarities between medial and lateral menisci observed by previous micro-anatomical analysis [Di Giancamillo, 2014], meniscal samples were used for the biochemical analysis and Western blot (see below). Whole menisci of all animals (n=24) were digested in papain (Sigma-Aldrich, Milan, Italy) for 16–24 h at 60°C: 125 mg/mL of papain in 100mM sodium phosphate, 10mM sodium EDTA (Sigma-Aldrich), 10mM cysteine hydrochloride (Sigma-Aldrich), 5mM EDTA adjusted to pH 6.5 and brought to 100mL of solution with distilled water. Later, the digested samples were assayed separately for proteoglycan and DNA contents. Proteoglycan content was estimated by quantifying the amount of sulphated glycosaminoglycans using the 1,9-dimethylmethylene blue dye binding assay (Polysciences, Inc., Warrington, PA) and a microplate reader (wavelength: 540 nm). The standard curve for the analysis was generated by using bovine trachea chondroitin sulphate A (Sigma). DNA content was evaluated with the Quant-iT Picogreen dsDNA Assay Kit (Molecular Probes, Invitrogen Carlsbad, CA) and a fluorescence microplate reader and standard fluorescein wavelengths (excitation 485 nm, emission 538 nm, cut-off 530 nm). The standard curve for the analysis was generated using the bacteriophage lambda DNA supplied with the kit.

2.2 Protein extraction and Western blot

For each experimental group (neonatal, young and adult), 8 specimens were processed (total nr=24). The samples were pulverized for 2 minutes at 3000 oscillations/min in a liquid nitrogen cooled dismembrator (Mikro-Dismembrator, Sartorius Stedim, Muggio, Italy). They were then homogenized in a buffer containing 50 mM TrisHCl, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP40, at pH 7.4, supplemented with protease inhibitor cocktail (Euroclone, Pero, Italy). They were centrifuged at 13000 g at 4°C for 10 minutes to discard cellular debris.

The total protein concentration was determined using bicinchoninic acid assay (BCA) (Euroclone), which exhibits a colour change of the sample solution from green to purple in proportion to protein concentration and the colour tone could then be measured. After addition of 0.05% bromophenol blue, 10% glycerol, and 2% β-mercaptoethanol, 50 µg of each sample were boiled and loaded onto 6% SDS–polyacrylamide gels. After gel electrophoresis, polypeptides were transferred to nitrocellulose filters (Sigma-Aldrich) and these membranes were incubated with 5% non-fat milk for 1 hour at room temperature to block non-specific sites. After this, all samples were probed using the following antibodies (Abs): anti-collagen 1 (1:500; no. NB600-408; Novus Biologicals, Littleton, CO, USA), anti-aggrecan (1:1000; BC-3, ab3773, Abcam, Cambridge, MA), and anti-GAPDH (clone GAPDH-71.1; Sigma-Aldrich) (and kept at room temperature for 2 hours. The filters were then washed and incubated for 1 hour always at room temperature with HRP-labelled secondary antibodies (1:5000; Bio-Rad, Hercules, USA). For the last step of the procedure, the blots were developed using a chemiluminescent substrate (WESTAR Nova 2011, Cyanagen, Bologna, Italy) to detect and characterize the proteins C, as previously described. In order to compare target protein expression levels between neonatal, young and adult menisci, it was necessary to use a loading control to normalize the data measuring the levels of GAPDH (a marker for total protein in each sample).

2.3 Micro anatomical analysis: Immunohistochemistry

Menisci (8 per group, n=24). were divided, through two radial-transversal cuts into three different parts: anterior horn, body and posterior horn. Subsequently, each part was subdivided into an inner and an outer part through a longitudinal cut (Figs. 2,3, left upper side).

Samples were then fixed in 10% (v/v) phosphate-buffered formaldehyde (n=24), dehydrated in a graded 50% (v/v), 70% (v/v), 95% (v/v) and 100% (v/v) ethanol series, embedded in paraffin and cut into 4 µm-thick consecutive sections. Each sample was used for two different IHC protocols. After rehydration, sections were washed in Phosphate Buffered Saline (PBS, pH 7.4) plus Triton for 5 minutes.; moreover,

block endogenous peroxidase using H2O2 for 8 minutes in a humidified chamber was applied and subsequently the slides were incubated with the first-step primary antiserum, 1:50 collagen I (1:500; no. NB600-408; Novus Biologicals, Littleton, CO, USA) for 24 h at 18–20°C in humid chamber, then washed in PBS, and subsequently treated with the rabbit EnVision system (Dakocytomation, Milan, Italy). The sections were then washed in PBS for 10 min and incubated with a 5 min 3,3' diaminobenzidine tetrahydrochloride (DAB)/hydrogen peroxide, which results in a brown precipitate at the antigen site. Counterstaining with Mayer haematoxylin for 1 minute allowed a better visualizing of the morphological structure; then, the slides were dehydrated and permanently mounted. For the second IHC, all steps for this protocol were identical to those of that previously described, except for few details: primary antibody was anti-aggrecan diluted in PBS at a concentration of 1:10 (ab3773, Abcam, Cambridge, MA) and prior to antibody processing and antigen retrieval with 0.01 Units Chondroitinase (Sigma-Aldrich) in PBS for 10 minutes was applied. Secondary antibody was EnVision mouse. The specificity tests for the antibodies were verified by incubating sections with: (i) PBS instead of the specific primary antibody; (ii) PBS instead of the secondary antibodies. The results of these controls were negative (i.e. staining was abolished). Photomicrographs were taken with an Olympus BX51 microscope (Olympus, Milan, Italy) equipped with a digital camera and final magnifications were calculated.

2.4 Biomechanical analysis

Menisci from the 3 swine groups were stored in saline solution NaCl 0.9% and frozen at -80°C until time of testing (8 per group, n=24). At least 24 hours before test execution, samples were taken to a temperature of -24°C and then completely thawed at room temperature (23°C). Each meniscus was prepared and cut in a different shape depending on the test run. Compression and circumferential traction tests were performed using EnduraTEC ELF® 3200 machine, equipped with a 220 or 22N load cell depending on the test and sample.

Compression test - For compression tests, specimens were obtained dividing each meniscus in the three portions: anterior horn, body and posterior horn. Subsequently, for each zone, a cylindrical part perpendicular to the femoral and tibial surfaces was cut using a die cutter:
(Fig. 4 left upper side). The diameter of the specimens was dependent on the original meniscus. Anterior horn, posterior horn and body of neonatal menisci were analysed as a whole, and no further divisions were made. Before testing, dimensional measurements on the specimens were made with a digital calliper (Mitutoyo Corp, Kanogawa, Japan, number of 06,081,911 series, accuracy class 1). The samples were inserted into a Plexiglas cell and PBS solution was added into the cell to avoid dehydration of the specimen. The thickness of all samples was measured from the position of the testing machine actuator, after imposing a preload of approximately 0.01N (young and neonatal menisci) or 0.1N (adult menisci). The sample was then subjected to a multi-ramp stress relaxation test, made of five increasing 4% strains

at a velocity of 0.1%/s, followed by stress relaxation to equilibrium for 600 s (adult and neonatal menisci) or 2000s (young menisci). The compressive Young modulus, EC, was obtained for each ramp from the equilibrium data as the ratio between values of relaxation stress and the corresponding values of strain. Circumferential traction test- For this test, we obtained representative fragments of the meniscus anterior horn, body and posterior horn. These fragments were cut out using a scalpel, trying to obtain samples with a shape as similar as possible to a parallelepiped (Fig. 4, left bottom line) and following the circumferential tensile force direction. Before testing, dimensional measurements on the specimens were made with the same digital calliper previously indicated. Width and thickness of each sample were obtained using the calliper, length instead was measured on the mounted specimen, considering as length the distance between the two grips after preload application. A preload of approximately 0.01N (young and neonatal menisci) or 0.1N (adult menisci) was applied. Neonatal menisci were considered only for the traction test of the central body because of the low dimension (i.e. anterior and posterior horn were used to block the sample in the machine). The specimens were subjected to a multi-ramp stress-relaxation test, made of four increasing 4% strains at a velocity of 0.1%/s, followed by stress relaxation to equilibrium for 1200 s. A custom made chamber filled with PBS was used to keep the samples hydrated during the test. The tensile relaxation Young moduli, ET, were determined for each ramp from the equilibrium data as the ratio between the relaxation stress value and the corresponding value of strain.

2.5 Statistical Analysis

Statistical analysis of the data (biochemical, Western blot and biomechanical results) were analysed with 2-ways ANOVA with age (neonatal, young and adult) and meniscal portions (anterior horn, body, posterior horn) as main factors. The statistical analysis was performed using the general linear model of the SAS (version 8.1, Cary Inc., NC). The individual meniscal samples were considered to be the experimental unit of all response variables. The data were presented as least squared means ± SEM. Differences between means were considered significant at $P < 0.05$ and highly significant at $P < 0.01$.

3. Results

3.1 Biochemical analysis

The biochemical analysis showed a significant decreasing cellularity from neonatal to young and adult ($P<0.01$ all comparisons, Fig. 1B), while GAGs revealed to be higher in adult and young vs neonatal ($P<0.01$, Fig. 1C) and GAGs/DNA ratio significantly increased with age from neonatal to young and adult ($P<0.01$ all comparisons, Fig. 1D).

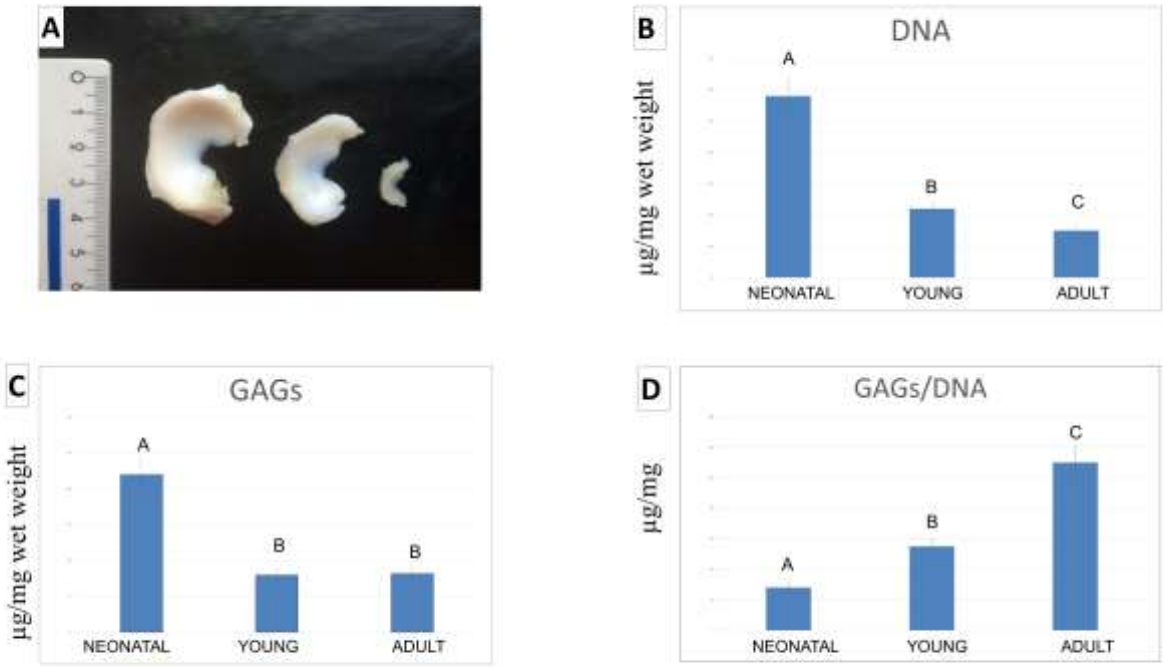

Fig 1 A) Macroscopic aspect and comparison of menisci belonging to the three classes of age. B-C-D) Differences in biochemical analyses of menisci belonging to the three classes of age: (B) DNA quantification; (C) GAGs quantification; (D) GAGs/DNA ratio. Quantification of DNA normalized to the wet weight (µg/mg). Quantification of GAGs normalized to the wet weight (µg /mg). Quantification of GAGs normalized to the DNA amount (µg /mg). Values with different superscripts (A, B, C) differ for $p < 0.01$.

3.2 Western Blot

Collagen I showed a progressive decrease in the different age groups: neonatal and young groups reveal a significantly higher level of collagen I if compared to adult one ($P<0.05$) (Fig. 2).

Aggrecan, contrary to what observed for collagen I, presented a statistically higher level in adults while in neonatal and young it showed a similar result ($P<0.01$) (Fig. 3).

3.3 Microanatomical analysis: Immunohistochemistry

Collagen type I inner part - Collagen type I showed fibers as well as nuclear immunopositivity in the inner part of anterior horn, body a posterior horn of all the different stages of maturation of the meniscus. Neonatal samples showed a higher cellularity with weak nuclear positivity of fibroblasts, but no specific pattern of organization of the fibers (Figs. 2-a,c,e) in the three considered zones. In young samples, a decreased cellularity was evident, although with maintained nuclear positivity in fibroblast cells of the three zones: in the posterior horn, a mature cellular phenotype, i.e, fibro-chondrocytes was evident. Fibers of collagen I with a linear trend were visible in the matrix (Figs. 2-a1,c1,e1).

Adult menisci presented an intense nuclear positivity, but also a more decreased cellularity. Noteworthy is the fact that the cellular phenotype in the anterior horn was less mature than that of the other two considered zones (Figs. 2-a2): a fibro-chondrocyte cellular phenotype was clearly evident in the body and posterior horn of adult meniscus (Figs. 2-c2,e2). In the matrix, circular and radial fibers were present and mature.

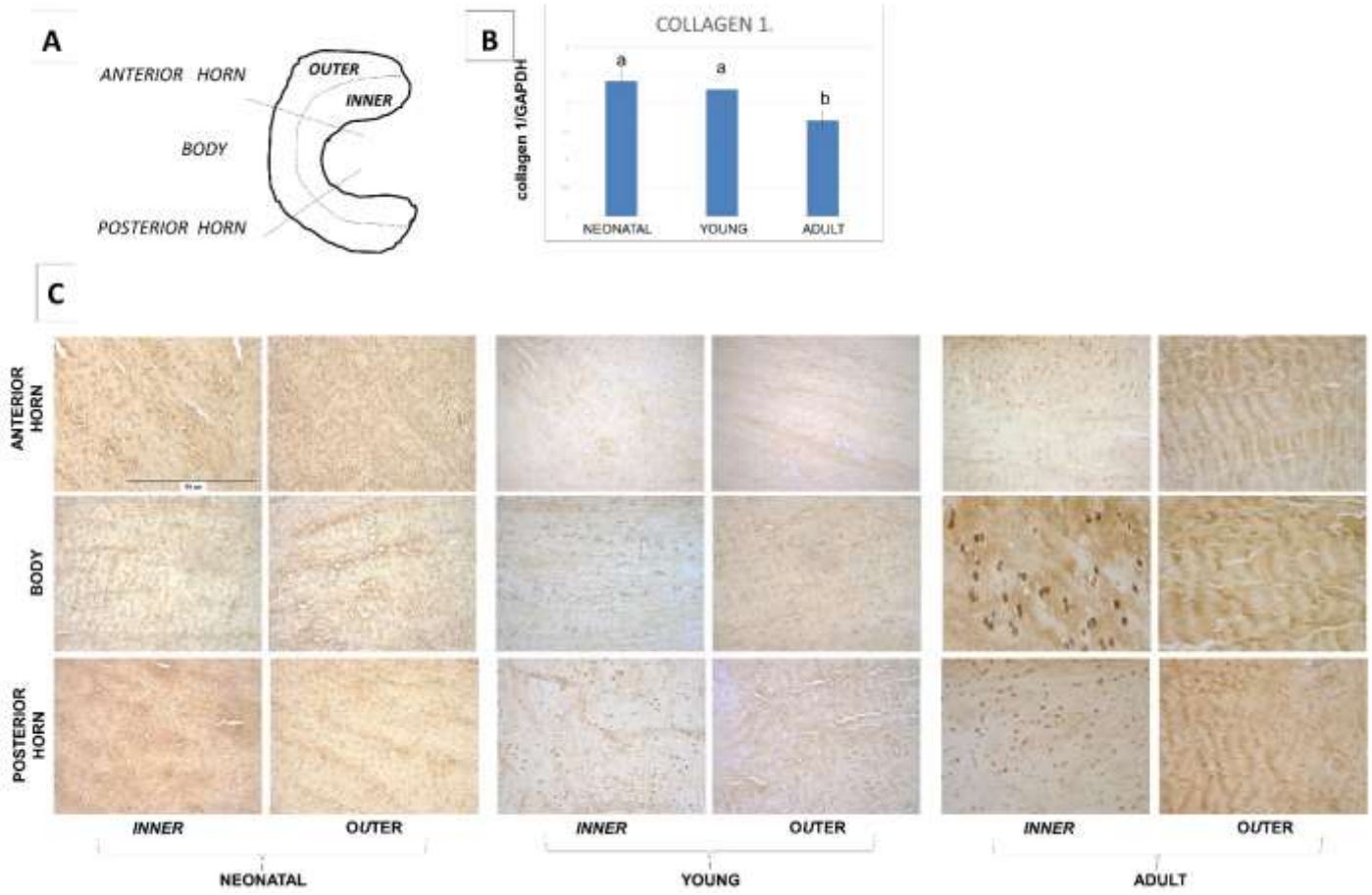

Fig. 2 From left to right upper sides: meniscal partition scheme which were subdivided transversally (in an anterior horn, a body and a posterior horn) and longitudinally, in an inner and an outer part.
Lower side: collagen type I immunohistochemistry. a-f) neonatal collagen I immunostaining in the inner (a, c, e) and outer (b, d, f) regions. a1-f1) young collagen I immunostaining in the inner (a1, c1, e1) and outer (b1, d1, f1) regions. a2-f2) adult collagen I immunostaining in the inner (a2, c2, e2) and outer (b2, d2, f2) regions. White arrow: fibroblast-like cells; black arrow: fibro-chondrocyte-like cells; asterisks: collagen I deposition.
Values with different superscripts (a, b) differ for $p < 0.05$.

Collagen type I outer part - In the outer part of the meniscus, the immunopositivity for collagen type I was particularly evident in all the considered zones and ages for both fibers and nuclear positivity (Figs. 2-b,d,f; -b1,d1,f1; -b2,d2,f2). In these samples, a decreasing cellularity during animal growth was evident, even though a more intense nuclear positivity was present in the adult (Figs. 2-b2,d2,f2).
In the outer part of the anterior horn, body and posterior horn, the meniscal matrix showed a higher degree of organization of the fibers. Additionally, the outer part presented a more precocious organization of the matrix fibers, mostly in the posterior horn (Figs. 2-f2).
Aggrecan inner part - Aggrecan revealed scarce nuclear immunopositivity in the inner part of anterior horn, body and posterior horn in all the different stages of maturation of the meniscus (Figs. 3-a,c,e; -

a1,c1,e1; -a2,c2,e2), although more intense signal was visible in the adult (Figs. 3 -a2,c2,e2). No evident fibers pattern was observed in the neonatal samples, whereas a marked maturation and organization of the fibers was evident in young and adult, especially related to the body where the cells were located along the traction forces (Figs. 3c1,c2). Cellularity decreased during growth.

Aggrecan outer part - Aggrecan immunopositivity reflected what had already been observed for the inner part (Figs. 3b,d,f; -b1,d1,f1; -b2,d2,f2). A more complex and mature fibers disposition in the matrix was evident, particularly in the adult samples (Figs. 3-b2,d2,f2).

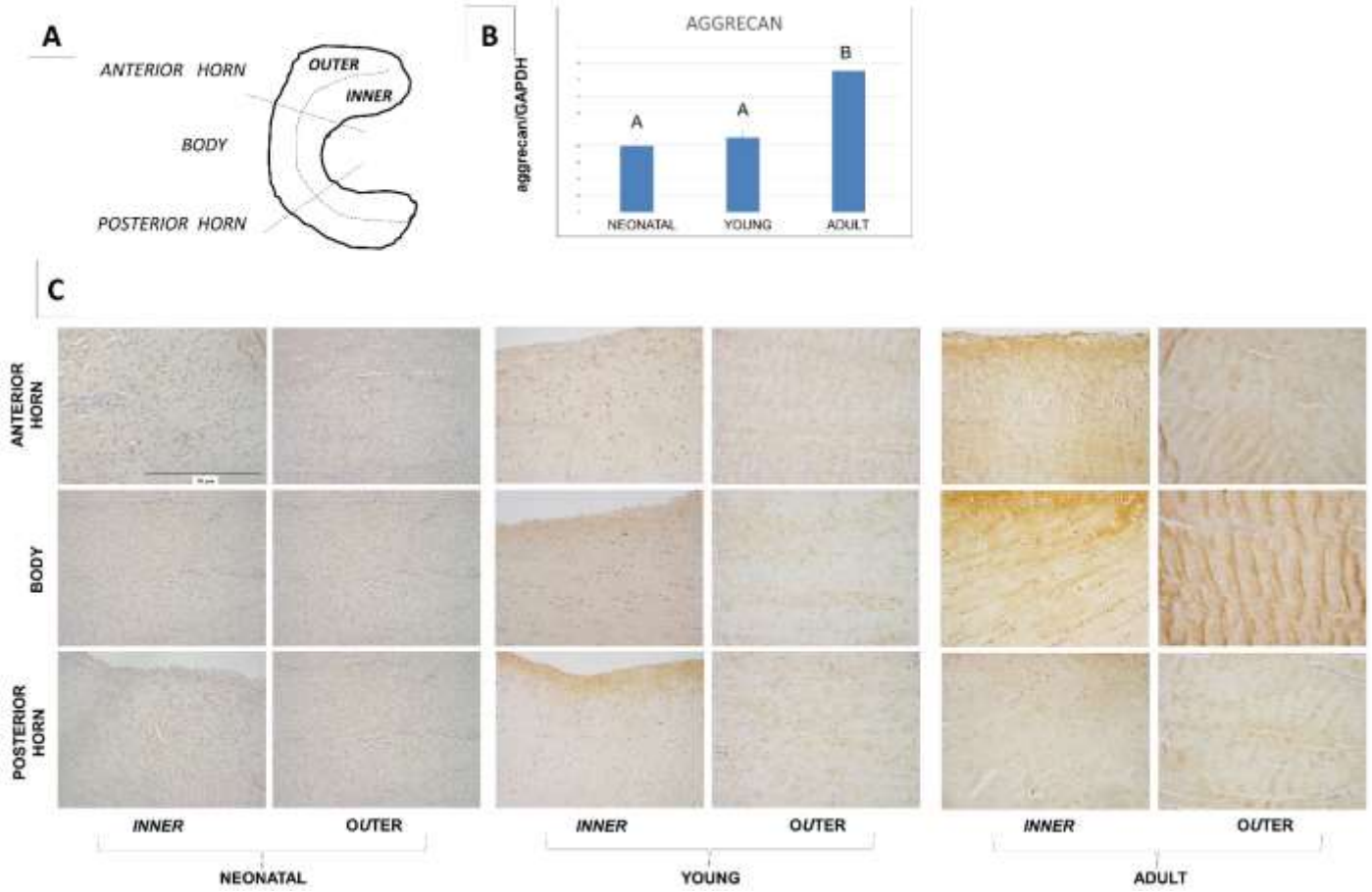

Fig. 3 From left to right upper sides: meniscal partition scheme which were subdivided transversally (in an anterior horn, a body and a posterior horn) and longitudinally, in an inner and an outer part;
Lower side: aggrecan immunohistochemistry. a-f) neonatal aggrecan immunostaining in the inner (a, c, e) and outer (b, d, f) regions. a1-f1) young aggrecan immunostaining in the inner (a1, c1, e1) and outer (b1, d1, f1) regions. a2-f2) adult aggrecan immunostaining in the inner (a2, c2, e2) and outer (b2, d2, f2) regions. White arrow: fibroblast-like cells; black arrow: fibro-chondrocyte-like cells; asterisks: aggrecan deposition.
Values with different superscripts (A, B) differ for $p < 0.01$.

3.4 Biomechanical analysis: compression and traction tests

We considered as representative value the Elastic Modulus (E) returned by each specimen during compression rate of 12%.

Compression test - Results of compression test are reported in Fig. 4. Pooled adult menisci revealed statistically higher values when compared to neonatal and young ($P<0.01$, Fig. 4A), and the same trend was observed in the anterior horn, central body and posterior horn ($P<0.01$ all comparisons, Fig. 4B).

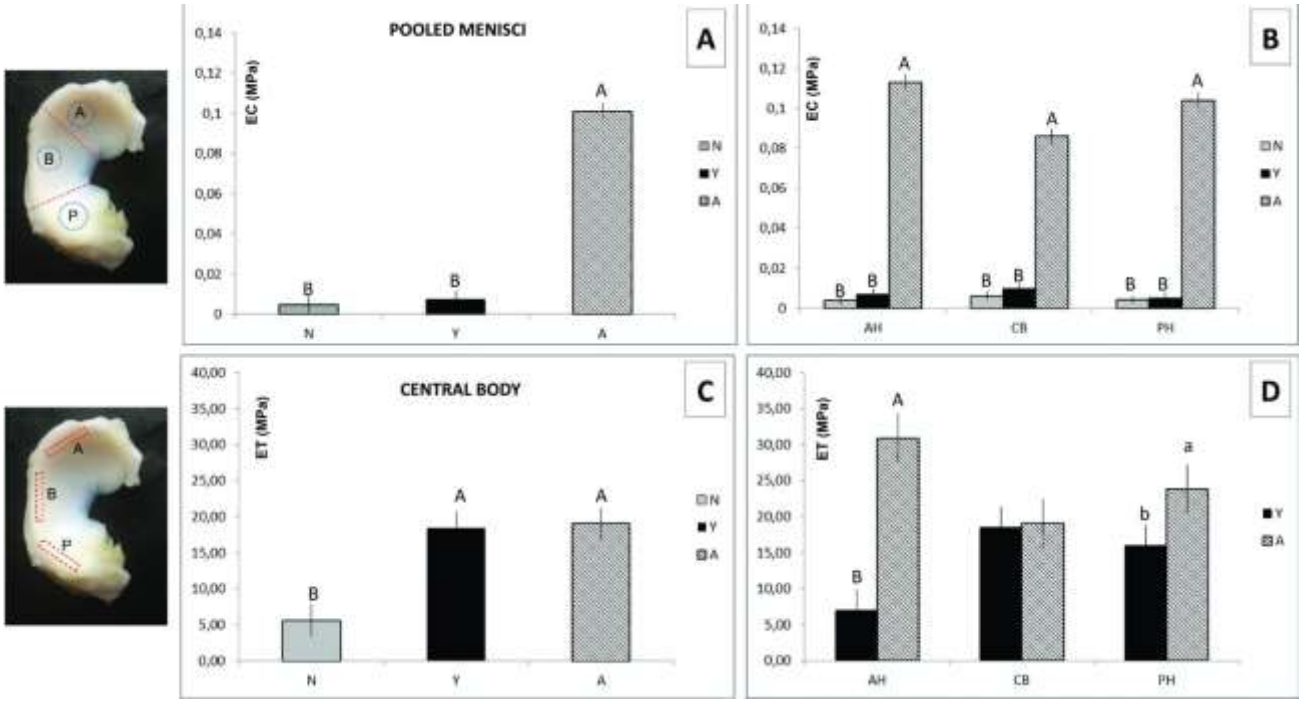

Fig. 4 Biomechanical results. Compression test results for pooled (A) and subdivided samples (B). Circumferential traction test results for pooled (C) and subdivided (D) samples. N: neonatal menisci; Y: young menisci; A: adult menisci; AH: anterior horn; CB: central body; PH: posterior horn. Values with different superscripts (A, B) differ for $p < 0.01$, and (a, b) for $p < 0.05$.

Circumferential Traction test - The ET of the central body increased during growth in age dependent-manner. When compared, adult and young menisci showed a similar result, whereas neonatal showed a significantly lower value with respect to all comparisons ($P<0.01$, Fig. 4C). Moreover, the mean value of the Elastic modulus of the young menisci showed significantly lower values compared to adult, both in anterior horn ($P<0.01$) and posterior horn ($P<0.05$), while no differences were observed in the central body (Fig. 4D).

4. Discussion

Results obtained in the present study show age-dependent properties of meniscus. Changes during animal growth are evident when immunohistochemistry, Western blot and biomechanical tests are evaluated and when results are interpolated. Cellularity, phenotype and protein expression, as well as fibres aggregation in the matrix are dissimilar in the three age categories analysed. These changes reflect the progressive maturation and hyper-specialisation of the meniscus.

The data obtained in this study will be discussed divided in age categories, in order to have a complete view for neonatal, young and adult swine.

Neonatal - Neonatal menisci express an immature profile with blood vessels and fibroblasts homogeneously distributed in the tissue, and these findings have been also reported by other authors, as observed in prenatal and postnatal maturation of meniscus in humans [Clark and Ogden, 1983] and in sheep [Meller et al., 2009]. After birth, no abrupt change in development occurs, but instead maturation

of meniscus requires gradual changes: decreasing in bloody supplies from the central to the peripheral margin, to the maturation in shape and to the conformation of articular surfaces. Quantitative analysis of the menisci, i.e. the Western blot, underlines a higher quantity of collagen I in the neonatal, but a minimal amount of aggrecan. The high prevalence of collagen I in the neonatal meniscus is indicative of a predominant fibroblast-like phenotype as observed by McDevitt et al. (2002) and Di Giancamillo et al. (2014).

Collagen I is homogeneously distributed in the matrix in the anterior horn, in the body and in the posterior horn and, perhaps, only in the posterior horn it is guessed a very first linear organisation of the fibres. Recently, Di Giancamillo et al. (2014) speculate that this precocious maturation of the posterior horn depends by the prevalent knee flexion that occurs in the swine during gestation and the first weeks of life. This hypothesis can explain the early differentiation of the posterior horn towards a fibro-cartilaginous tissue that is then followed by a marked maturation of the anterior horn in response to the increasing extension of the knee that occurs with growth. Collagen I is a typical fibroblast secretion product and its high prevalence in the neonatal/young meniscus is indicative of a predominant fibroblast-like phenotype. This result is in agreement with other investigations about cellular phenotype in the meniscus [McDevitt et al., 2002; Di Giancamillo et al., 2014]. Fibroblast-like cells have the ability to differentiate when specific stimuli are present [Guilak et al., 2009]. The absence of this differentiation, probably due to an inconsistent compressive stimulus in this age category, is reflected by the aggrecan distribution. In fact, aggrecan in neonatal meniscus follows what was already said for the collagen I, even if it occurs in much lower quantities. All these aspects are reflected in the mechanical properties of neonatal meniscus. The Elastic modulus in compression (Ec) is comparable to the results obtained in young animals (see below). These results indicate a small resistance to compressive forces, with a high grade of deformation of the meniscus when loaded. However, regarding the elastic modulus in traction (Et), neonatal meniscus shows a lower resistance to traction along the circumferential fibers respect to the other categories, naturally these results are comparable only for the meniscal body, due to the technical limitation exposed previously.

A mature organisation of the extracellular matrix is necessary to increase the ability to counteract the stress stimuli; therefore, in the neonatal, there is not the fundamental structure required to perform this function. Considering the resistance and the deformability, no differences are found within anterior horn, body and posterior horn of neonatal meniscus.

Young - Menisci obtained from young swine present characteristics halfway between the neonatal and the adult swine. However, in the young meniscus, a radial pattern of organization of collagen I in the matrix is evident considering the results of the immunohistochemistry evaluation. This is particularly true in the central body and in the posterior horn. Maturation and organization are also evident when aggrecan is considered. In the central body of the young meniscus, cells are particularly abundant and disposed in

a way that reflects the tensile force lines: cells are more present and more active in aggrecan expression where compression forces are more intense. Even the fibres in the extracellular matrix are present with some grade of radial and circumferential organization; again, it is more evident in sections that are representative of the central body and of the posterior horn. Quantitatively, aggrecan is comparable to neonatal meniscus. Looking to compression test, the Elastic modulus in compression (EC) in the young animals is highly similar to the neonatal one. This means that, despite the initial distribution of aggrecan in the matrix, the young meniscus is not mature enough to resist to the deformation caused by the induced compression. Different results are obtained in traction: the young meniscal body shows an increased resistance respect to the neonatal one, a resistance similar to the adult meniscal body. But, if anterior and posterior horns are considered, young meniscus shows a lesser resistance to traction respect to adult one, indicating that collagen I organization, although started, is not already functional in these areas. All these considerations indicate that young meniscus in swine represents the beginning of matrix maturation that is concomitant with a cellular phenotype differentiation observed at the same age in a recent study on swine [Di Giancamillo et al. 2014]. However, its features are still too immature to express a functional biomechanical behaviour and to manifest an organized ultrastructure.

Adult - The adult samples were characterized by a higher loading pressure on the menisci. The specific characteristics of menisci in adult animals define a mature ultrastructure and cellular differentiation, and the deformability of the samples is significantly reduced when they are subjected to stress analysis. There is an evident quantitative decrease in collagen I expression. This is due to the switch in cellular phenotype from fibroblast-like cells to mature fibro-chondrocytes [Melrose et al., 2005; Valiyaveettil et al., 2005]. Di Giancamillo et al. (2014) described changes from young to adult meniscus in swine model, defining how the fibres composition along with the anterior to posterior aspect were quantitatively characterized by an increasing production of GAGs and collagen II accompanied by a reduction in collagen I deposition. Collagen I is quantitatively inferior to the collagen I found in neonatal and young animals, although it shows an increased deposition in the extracellular matrix and a complex pattern of organization. Aggrecan instead is significantly superior than the two age categories. Aggrecan has the ability to resist compressive loads: it retains considerable quantities of water and its osmotic properties are necessary to counteract compressive loads on the tissue [Kiani et al. 2002]. Aggrecan is also distributed in the matrix describing radial and circular pattern, probably disposed following force lines. There are not particular regional differences in the aggregation of collagen fibres from the anterior to posterior horn. Aggrecan expression and organization, instead, are superior in the body. Cellular phenotype is different from what was seen in young and neonatal samples: cells in the inner part of adult meniscus show an increased volume and a spherical shape, an increased nuclear immunopositivity for both collagen I and aggrecan. They are arranged in isogenous groups and are surrounded by pericellular matrix, in agreement with meniscal fibrochondrocytes described in other studies [Hellio Le Graverand et al. 2001; Gunja et al. 2007; Di

Giancamillo et al. 2014]. The cellularity is highly decreased, as Bland and Ashhurst (1996) have already observed in 8 months old rabbits, but the cells were very active in protein expression. This means that in the inner part of mature menisci there are specialized cells secreting components of the extracellular matrix. Melrose et al. (2005) deal with a comparative spatial and temporal localisation of aggrecan and type I collagen in the ovine meniscus from 2 days to10-year-old. This report shows that aggrecan is strongly immunolocalised to the tip of the inner zone, where also collagen II is found. Type I collagen, however, is uniformly immunolocalised throughout the outer, middle and inner zones of the meniscus at all time points examined, confirming the fibrocartilaginous classification of this tissue type.

The use of two-dimensional (2D) imaging techniques, i.e. immunohistochemistry, to evaluate a highly three-dimensional (3D) structure is an obvious limitation. Nevertheless, our investigation about fibres distribution in the adult meniscus of pig, although bi-dimensional, can find confirms in other researches. Many studies currently use very sophisticated imaging techniques to deeply investigate tissues in three-dimensions. These are often just experimental analyses, but the results obtained are comforting and surprising. Zhang et al. (2014) observed the orientation of the collagen fibres in adult porcine meniscus, while Andrews at al. (2013) investigated bovine meniscus. They both demonstrated and reproduced three-dimensional images of fibres and their different orientation between the superficial and internal portions, confirming the disposition described in two-dimension analyses. Compression tests revealed that the adult meniscus greatly resists to deformation, i.e. it has a high value of EC. This ability, as already said, is due to the characteristics of the substances distributed in the extracellular matrix and aggrecan has a main role in this process. Traction tests have also showed the ability of the adult meniscus to resist to tensile forces. Adult meniscus has a high value of elastic modulus, with a decreasing trend from the anterior horn to the posterior horn. This property is dependent by collagen I organisation in the matrix. During traction and compression, the elastic modulus describes the same profiles: in compression, if the material analysed is hardly deformable, the elastic modulus is high, and this indicates that the material can resist to compression stimuli. In traction, the force applied tends to elongate the material, that is, if the material can resist to tensile force, the elastic modulus is high. This is exactly what our traction results revealed. The elastic modulus in the adult meniscus is elevated and this indicates its ability to counteract tensile forces, mainly in its anterior horn.

The mature extracellular matrix found in the adult meniscus confers to the latter the strength properties verified during biomechanical tests. Collagen I fibres are slightly more expressed in the anterior horn and in the central body, so it is the aggrecan.

Anterior horn is the most solicited part in normal stifle during locomotion, and so is also the meniscal body. Higher differentiation of these parts of the meniscus is consequence of stifle kinematics. Sweigart et al. (2004; 2005) and Sandmann et al. (2013) subdued to biomechanical tests menisci from different animal species. Chia et al. (2007) instead have defined biomechanical properties of human meniscus. In

all these studies, regional differences in meniscal response were found both in compression and in traction, and the anterior horn was recognised as the part with the highest stiffness. Sweigart and his team analysed compressive properties of meniscus in six animal models (baboon, bovine, canine, human, leporine and porcine) [Sweigart et al. 2004] and topographical variations among the porcine meniscus subdues to biomechanical tests [Sweigart and Athanasiou, 2005]. They found that significant intra- and interspecies variations in biomechanical properties among the different regions of the meniscus are present. The femoral anterior portion of medial meniscus was found as the one having the highest stiffness in all the species while the tibial posterior region showed the lowest values. Sandman et al. (2013) compared resistance to compression in bovine, ovine and porcine menisci and artificial scaffolds. Chia et al. (2007) investigated elastic modulus in human meniscus during compression. Also, in this study, the anterior region showed the greater stiffness whereas the posterior region was the weaker. Also, in our investigation regional differences resulted, but no remarkable difference of the anterior horn was found in compression, while in traction this portion showed increased stiffness in comparison to other parts. Considering the whole data, the results obtained in the present study are in agreement to what is present in literature. With this study, we demonstrated that in swine meniscus clear changes follow the meniscal maturation during growth: starting with an immature cellular and fibre pattern (neonatal) to the mature organised and differentiated meniscus of the adult. The changes in swine meniscus reflect themselves in different biomechanical behaviours: the increasing of stiffness is evident, both against compression and to tensile force. Hyper-specialisation of meniscus is obtained sacrificing its vascularization and regenerative capacity: there is a clear correlation between cellular differentiation and meniscal maturation with progressive reducing of blood supply. Considering age-dependent characteristics, young category represents the transition phase, where first step of maturation is made. Young samples were obtained to 1-month-old animals. In pig farm, this age coincides with the weaning and the start of the production phase. This is the moment when these animals start to increase their weight and contemporary have the possibility to walk. Weight and locomotion are the stimuli that boost the meniscus maturation.

5. Conclusion

An increasing evidence has revealed that a different array of additional environmental factors contributes to the overall control of stem cell activity, focusing on the importance of the extracellular matrix [Boudreau and Jones, 1999; Streuli, 1999; Guilak et al. 2009]. The extracellular matrix (ECM) is an ‘informational’ entity in the sense that it receives, imparts and integrates structural and functional signals [Bissel and Barcellos-Hoff, 1987]. The way that this “dynamic reciprocity” between cells and ECM is actuated is still not perfectly known, but many studies have investigated the matrix in this sense [Lin and Bissel, 1993; Streuli, 1999; Boudreau and Jones, 1999; Tan and Cooper, 2011; Mauk and Burdick, 2015].

Data on the important influence that the extracellular matrix has on cell fate are still growing. It is now clear that ECM-based control of the cell may also occur through multiple physical mechanisms, such as ECM geometry at the micro- and nanoscale, ECM elasticity, or mechanical signals transmitted from the ECM to the cells [Guilak et al. 2009].

These studies show that physical interactions with the ECM significantly influence the cell behaviour, and that it can interact with chemical (i.e., composition), molecular (i.e., soluble mediators), or genetic (cell-type) factors in order to regulate cell fate.

A greater understanding of its structural characteristics can provide useful insights into the overall understanding of the development of the meniscus itself. Studies about meniscal maturation in animal models could represent landmarks for the development of artificial scaffolds.

The present study demonstrated that in swine meniscus clear changes follow the meniscal maturation during growth: starting with an immature cellular and fiber pattern (neonatal) to the mature organised and differentiated meniscus of the adult.

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Conflict of Interest Statement

The authors confirm that there are no conflicts of interest.

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3.2. DECORIN AGE-RELATED VARIATIONS IN THE DISTRIBUTION OF PIG EXTRACELLULAR MATRIX MENISCUS

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Abstract:

Menisci act like a shock absorber and transmits load across the tibiofemoral joint by increasing congruency during movements or body weight load. This leads to decreasing the resultant stress on the articular cartilages. The meniscus has a dense extracellular matrix (ECM) composed of water, different types of collagens, and proteoglycans, such as decorin, aggrecan and biglycan. Decorin (DCN) regulates collagen fibrillogenesis acting on collagen fibrils diameter and fibrils orientation to achieve the proper assembly of its network. This work investigates the spatial disposition of this fundamental protein in pig meniscus' matrix by immunohistochemistry and western blot analysis. DCN shows an increasing in its amount trend, moving from neonatal to adult pig menisci. Adult meniscus, in porcine species, is the only one that could be considered fully mature and functional, and, even if an increasing trend is seen, no precise phenotypical switch points are seen in the age stages considered in this study.

Keywords: decorin, swine, pig, menisci, age-related variations, matrix

1.Introduction:

The different shapes of the two ends of the knee joint, the convex femoral and the flattened tibial condyles, make essential the presence of the wedge-shaped menisci to allow the correct function of this joint [Fox et al., 2012; Peretti et al., 2019]. Meniscus acts like a shock absorber and transmits load across the tibiofemoral joint by increasing congruency during movements or body weight load. This leads to decreasing the resultant stress on the articular cartilages [Fox et al., 2012]. Other functions of the meniscus are inherent to the distribution of nutrients within the articular space, stability, lubrication and proprioception to the knee joint [Fox et al., 2012]. These functions are possible due to the peculiar structure that characterizes the meniscus: a triangular shape after transversal section. The apex directed towards the intercondylar notch is avascular in the adulthood and for this reason it is called “white-white” zone. The external and wider border is vascularized throughout all the life and it is called “red-red” zone [Di Giancamillo et al., 2017]. Between these two, not well outlined, exists an intermediate area with midway characteristics, called white-red zone. At each area corresponds a different cellular phenotype expression and a peculiar organization of the biochemical components. The inner portion of the meniscus is characterized by round-shaped cells that resemble and act like the chondrocytes, the fibro-chondrocytes [Di Giancamillo et al., 2016; Verdonk et al., 2005]. On the other hand, the oval cells of the outer zone are classified and act as fibroblasts [Verdonk et al., 2005]. A third cellular population characterized the most superficial zone of the meniscus and seems to have regenerative capacity and act as specific progenitor cells [Van der Bracht et al., 2007]. Meniscus has a fibrocartilaginous nature and it is composed of an extracellular matrix (ECM) mainly composed of water (72%) and collagen (22%) [Ghadially et al., 1983]. Collagen is the main fibrillar component of the meniscus and is the primarily responsible for the tensile strength of the meniscus [Herwig et al., 1984]. Its distribution and amount depending on the considered meniscal area. The inner zone is composed exclusively of two different types of collagen (70% by dry weight): types II (60%) and I (40%) [Cheung, 1987]. On the contrary, the outer zone is characterized by type I collagen as major constituent (80% of the total dry weight), in association with some variants (e.g., type II, III, IV, VI, and XVIII) that represent less than 1% of the total dry weight [Fox et al., 2012]. Collagen follows different patterns of distribution in base of the considered zone. Two different configurations mainly represent these patterns: the circumferential and the transversal ones. An abundance of the circumferential disposition was seen in the femoral surface, while a radial disposition of the fibres characterises the tibial surface in the swine meniscus. These radially positioned “tie” fibers woven between the circumferential fibers to provide structural integrity [Bullough et al., 1970; Yasui, 1978; Aspden et al., 1985; Beaupre et al., 1986; Fithian et al., 1990; Skaags and Mow., 1990; Arnoczky et al., 1998; Fox et al., 2012; Peretti et al., 2019] and allow the dissipation of the forces through the meniscal ligaments [Voloshin and Wosk, 1983]. Other meniscal constituents include glycosaminoglycans (17%), adhesion glycoproteins (<1%), and elastin (<1%) [Herwig et al., 1984; Makris et al., 2011]. The proportion

of each component varies according to age or the presence of pathological condition [Sweigart and Athanasiou, 2001]. Meniscal glycosaminoglycans including aggrecan, decorin, biglycan and perlacan, withstand to compressive forces. Aggrecan is more abundant in the inner regions than the outer regions of canine, porcine and ovine menisci [Scott et al., 1997; Valiyaveettil et al., 2005; Melrose et al., 2005]. Glycosaminoglycans are more abundant in the femoral portion than the tibial portion in the porcine meniscus [Peretti et al., 2019]. Decorin (DCN), so named for its ability to bind to and "decorate" collagen fibrils, has a core protein of approximately 38kDa, is a small leucine-rich proteoglycan (SLRP) which binds 10 leucine-rich repeat sequences. It binds to and regulates collagen fibrillogenesis acting on collagen fibrils diameter and fibrils orientation to achieve the proper assembly of its network [Boskey and Robey, 2013]. Absence of decorin, in tendons, results in irregular collagen fibril morphology with fusion of adjacent fibrils [Danielson et al., 1997]. The complex composition and organization of meniscal extracellular matrix (ECM), necessaries to provide the fundamental load-bearing and stress- dissipating properties of this tissue makes challenging to fabricate an engineered graft for its repair. A full understanding of the composition of the extracellular matrix of healthy meniscus is required to evaluate the degeneration that occurs in joint disease and the complex environment in which an engineered meniscal substitute would need to function [Vanderploeg et al., 2012]. Furthermore, it must be considered that the extracellular matrix may influence cells triggering a different cellular response. For this reason, many tissue engineering attempts have been made for tissue repair or regeneration by working on different ECM substrates. Even if pig's meniscus is often used like model for the human one [Proffen et al., 2012; Zhang et al., 2014; Di Giancamillo et al., 2014; Deponti et al., 2015] works regarding the different spatial distribution of DCN within its matrix are inexistent. This work aims to find out how DCN is distributed in the swine meniscus and how this distribution varies during growth.

2. Materials and Methods:

2.1 Study design:

For the aim of this study, we obtained menisci from a local slaughterhouse, which breeds swine Landrace x Large White. Knees of adults (~8-month old, weight 85-90 kg), young (1-month old, weight 10-12 kg), and neonates (stillbirths or dead in peripartum) were collected. The joints were dissected to isolate lateral and medial menisci. Capsular tissue and ligaments were gently removed, the menisci were stored in saline solution NaCl 0.9% and refrigerated. No animal has been sacrificed for the purposes of this study; the research was approved by the Ethic Committee of the University of Milan (OPBA, 58/2016).

2.2 Micro anatomical analysis: Immunohistochemistry

Given the morphological similarities between medial and lateral menisci observed by previous researches [Di Giancamillo et al., 2014; Zhang et al., 2014], only the lateral menisci were analysed (8 per group, n=24).

Menisci were divided, through two radial-transversal cuts, into three different parts: anterior horn, body and posterior horn. Subsequently, each part was subdivided into an inner and an outer part through a longitudinal cut (Fig.1). Samples were then fixed in 10% (v/v) phosphate-buffered formaldehyde (n=24), dehydrated in a graded 50% (v/v), 70% (v/v), 95% (v/v) and 100% (v/v) ethanol series, embedded in paraffin and cut into 4 µm-thick consecutive sections. After rehydration, sections were washed in Phosphate Buffered Saline (PBS, pH 7.4) plus Triton for 5 minutes.; moreover, block endogenous peroxidase using H2O2 for 8 minutes in a humidified chamber was applied and subsequently the slides were incubated with the first-step primary antiserum, 1:500 decorin (rabbit polyclonal, no. ABIN2783275; Antibodies-online, Aachen, Germany) for 24 h at 18–20°C in humid chamber, then washed in PBS, and subsequently treated with the rabbit EnVision system (Dakocytomation, Milan, Italy). The sections were then washed in PBS for 10 min and incubated with a 5 min 3,3' diaminobenzidine tetrahydrochloride (DAB)/hydrogen peroxide, which results in a brown precipitate at the antigen site. Counterstaining with Mayer haematoxylin for 1 minute allowed a better visualizing of the morphological structure; then, the slides were dehydrated and permanently mounted. Secondary antibody was EnVision rabbit. The specificity tests for the antibodies were verified by incubating sections with: (i) PBS instead of the specific primary antibody; (ii) PBS instead of the secondary antibodies. The results of these controls were negative (i.e. staining was abolished). Photomicrographs were taken with an Olympus BX51 microscope (Olympus, Milan, Italy) equipped with a digital camera and final magnifications were calculated.

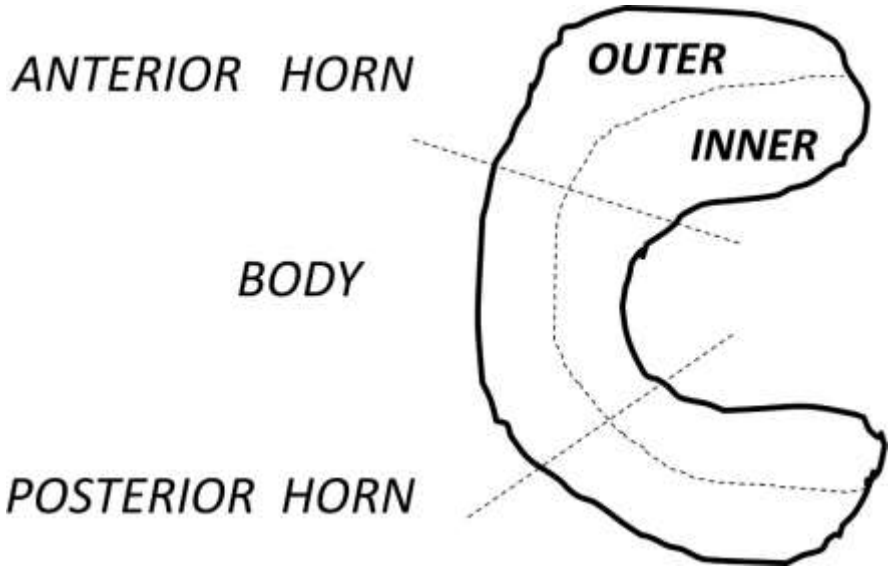

Fig 1 Schematic description of the cuts performed for the immunohistochemistry evaluation.

2.3 Western blot analysis

For each experimental group (neonatal, young and adult), 8 specimens were processed (total n=24). The samples were pulverized for 2 min. at 3000 oscillations/min in a liquid nitrogen cooled dismembrator (Mikro-Dismembrator, Sartorius Stedim, Italy). They were then homogenized in a buffer containing 50

mM Tris–HCl, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP40, pH 7.4, supplemented with protease inhibitor cocktail (Euroclone, Pero (MI) Italy) and centrifuged at 13000 g at 4°C for 10 min. to discard cellular debris. Protein concentration in the extracts was determined using a BCA protein assay (Euroclone). After addition of 0.05% bromophenol blue, 10% glycerol and 2% b-mercaptoethanol, 50 ug of each sample was boiled and loaded onto 8% SDS–polyacrylamide gels. After electrophoresis, polypeptides were electrophoretically transferred to nitrocellulose filters (Sigma-Aldrich, Milan, Italy); the membranes were incubated with 5% non-fat-milk for 1 hr at room temperature to block the non-specific sites and then probed for 2 hrs at room temperature using anti-decorin (1:500; rabbit polyclonal, no. ABIN2783275; Antibodies-online, Aachen, Germany), anti-GAPDH (1:1000, clone GAPDH-71.1) and kept at room temperature for 2 hours. The filters were then washed and incubated for 1 hour always at room temperature with HRP-labelled secondary antibodies (1:5000; Bio-Rad, Hercules, CA, USA). For the last step of the procedure, the blots were developed using a chemiluminescent substrate (WESTAR Nova 2011, Cyanagen, Bologna, Italy) to detect and characterize the proteins C, as previously described. In order to compare target protein expression levels between neonatal, young and adult menisci, it was necessary to use a loading control to normalize the data measuring the levels of GAPDH (a marker for total protein in each sample) Immunoreactivity was detected by chemiluminescence autoradiography according to the manufacturer's instructions (Biorad, Milan, Italy), and the images were scanned. The optical intensities of the protein bands of interest were determined densitometrically using Scion Image software (Scion Corporation, Frederick, MA).

2.4 Statistical analysis

Statistical analyses of the Western blot essay results were analysed with a 2-ways ANOVA, with age (neonatal, young and adult) as main factor. The statistical analysis was performed using the general linear model of the SAS (version 8.1, Cary Inc., NC). The individual meniscal samples were considered to be the experimental unit of all response variables. The data were presented as least squared means ± SEM. Differences between means were considered significant at $p<0.05$ and highly significant at $P < 0.01$.

3. Results:

3.1 Immunohistochemistry:

Decorin shows a principally pericellular immunopositivity and increases with age (Fig. 2). It is present since the neonatal phase, even if with differences in the distribution: posterior horn (Fig. 2- e,f) presents a higher immunopositivity respect to the anterior horn (Fig. 2-a,b) and body (Fig. 2-c,d) portions. Differences in the distribution of decorin is also seen between inner and outer regions of posterior horn (Fig. 2- e,f), with an increased immunopositivity in the first (Fig. 2e) respect to the latter (Fig. 2f). In young and adult meniscus, decorin shows a more homogeneous distribution among anterior horn, body and

posterior horn (Fig. 2- a1-f1 and a2-f2). However, an increased immunopositivity is still present in the inner portion (Fig. 2- a1,c1,e1) of the young meniscus when compared to the outer one (Fig. 2- b1,d1,f1). Adult samples show no differences between inner (Fig. 2- a2,c2,e2) and outer (Fig. 2- b2,d2,f2) portions with regard to the distribution of decorin. The pattern of distribution change between the two regions: in the inner part decorin shows a pericellular distribution (Fig. 2-a2,c2,e2; black arrows) while in the outer it is mainly linked to the presence of transversal fibers (Fig. 2-b2,d2,f2; white arrows).

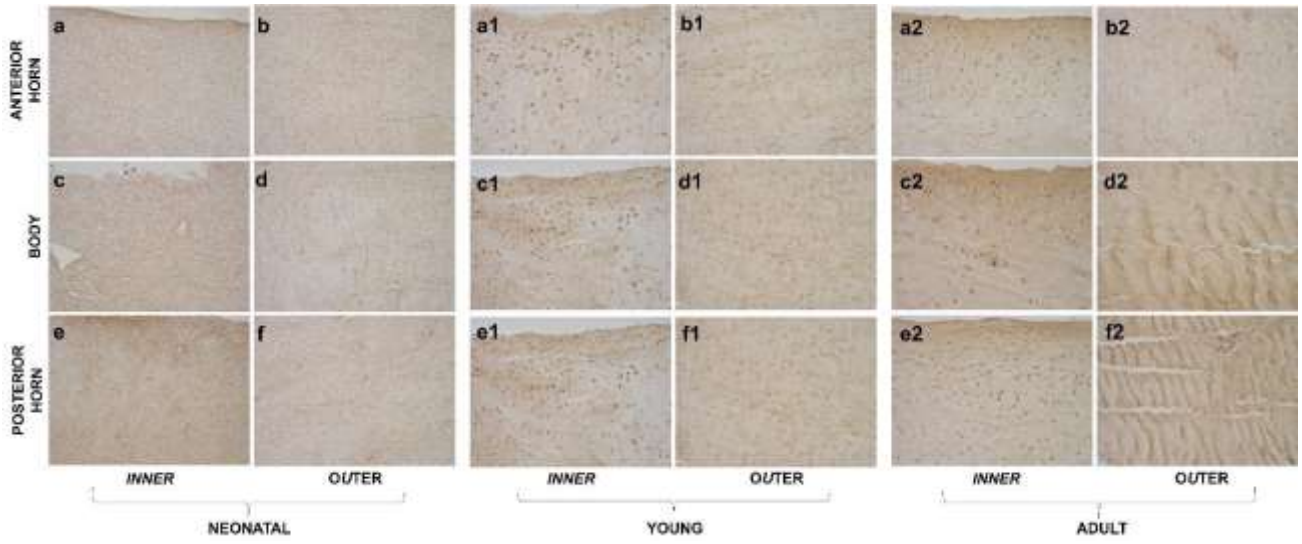

Fig 2 Decorin expression in the different portions of the meniscus and at different age: a-f: neonatal meniscus; a1-f1: young meniscus; a2-f2: adult meniscus; a,c,e: inner part of the neonatal meniscus; b,d,f: outer part of the neonatal meniscus; a1, c1,e1: inner part of the young meniscus; b1,d1,f1: outer part of the young meniscus; a2,c2,e2: inner part of the adult meniscus, b2,d2,f2: outer part of the adult meniscus. Immunopositivity is marked in brown and is principally pericellular. Scale bar is the same as located in -a.

3.2 Western blot analysis:

Western blot in neonatal, young and adult animals, revealed the same trend of expression as immunohistochemistry, characterized by an increasing trend of the amount of this proteoglycan with age (Fig. 3B). In particular, adult menisci show the highest quantity of decorin when compared with both young and neonatal ones ($P<0,001$, for both comparisons; Fig. 3B).

4. Discussion:

In pig meniscus, decorin shows an age-related distribution. DCN is present since the earliest stage of development of this structure (in this case, at birth). Nevertheless, its distribution in neonatal specimens shows some peculiarity regarding the area of the meniscus that is taken into account: the inner portion of the posterior horn is the only one in which a satisfactory immunopositivity is observed at this time stage. This result agrees with a previous work of our research group [Di Giancamillo et al., 2014] in which a precocious maturation of the posterior horn of the meniscus, due to the prevalent knee flexion that occurs in the swine model during gestation and the first weeks of life of the swine, is hypothesized.

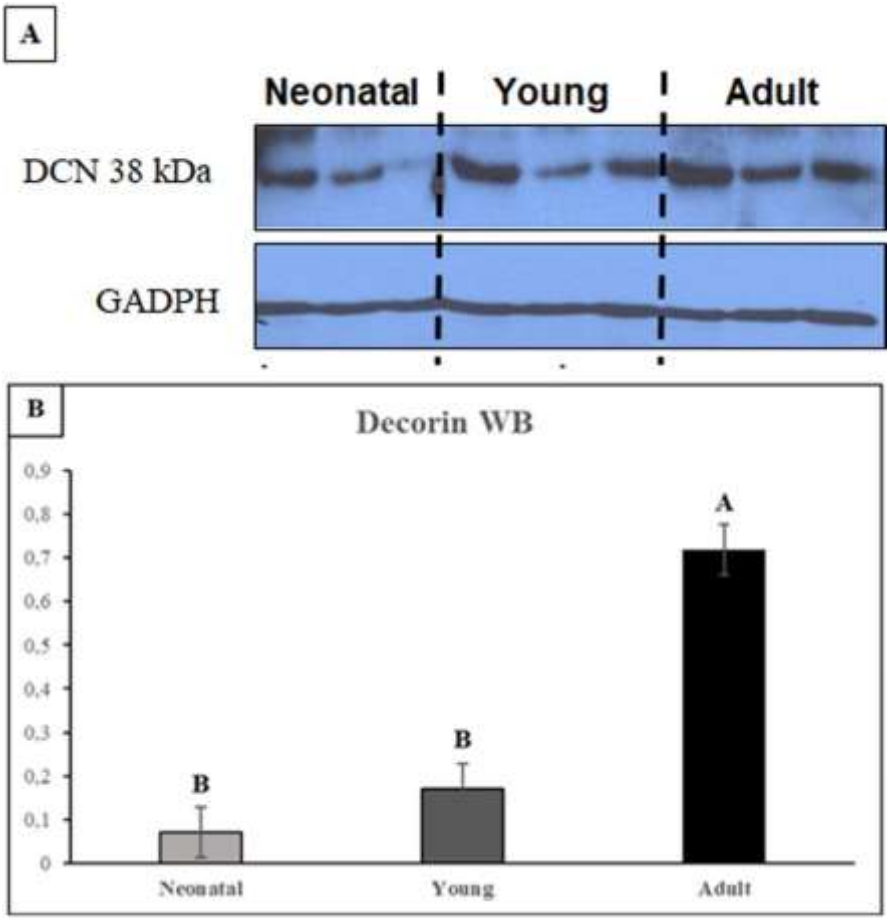

Fig 3 Western blot analysis. A: Western blot lines. The discrete, 38 kDa, lines that correspond to the adult meniscus show a grossly increasing deposition of secondary antibody, compared to the lines of the other two stages of age. GADPH as control; DCN: decorin. B: The graphic shows the quantification of Western blot analysis, performed by means of densitometry analysis through dedicated software (Imagej). Values with different subscripts (A, B) for $p < 0.01$.

The inner portion is richer of decorin even in the meniscus of young animals, as for all the other proteoglycans, as seen in previous observations in this meniscal portion of different species, like pigs [Scott et al., 1997; Peretti et al., 2019], canine [Valiyaveettil et al., 2005], ovine [Melrose et al., 2005] and human [Makris et al., 2011].

In the adult meniscus the differences between inner and outer region distribution of decorin is not so demarcated as in the precocious age stages, and so the distribution of this proteoglycan results more homogeneous. Only the pattern of distribution seems to change: in the inner portion the distribution is strictly linked to the cell's presence, being mainly pericellular, while in the outer region the distribution of decorin follows principally the fibers trend. This peculiar pattern shows some affinity with what was observed, intriguingly in the juvenile (2-4 weeks), bovine meniscus by Vanderploeg and collaborators [Vanderploeg et al., 2012]. The precocious distribution, swine-adult-like, of this proteoglycan in the bovine model may be due to a different biomechanical stimulus given by the different weight of pigs and bovines at 1 month. This hypothesis, of a distribution linked to the weight, seems to be confirmed by the observation of Kavanagh et al. in 3 weeks and 8 months old rabbit's menisci: initially, decorin is present

throughout the fibrocartilage, but by 8 months its distribution is more in the periphery of the fibrocartilage [Kavanagh and Ashhurst, 2001].

DCN interacts with multiple collagens to create functional bridges between the pericellular matrix and the surrounding extracellular matrix and, in association with collagen type VI, is essential in cellular resistance to deformation [Twomey et al., 2014].

In human being, age-related changes in the composition and structure of collagens, proteoglycans, glycosaminoglycan chains and non-collagenous proteins have been reported: decorin and biglycan analysis showed that decorin is the dominant, endogenous, non-aggregating proteoglycan in the adult meniscus and cartilage [Sampaio et al., 1988; Roughley and White, 1992] but in immature articular cartilage biglycan was present at much higher concentrations than decorin [Roughley and White, 1989]. In the present study biglycan was not considered but, given its mutually exclusively relation with decorin, as seen in rabbit and human, an influence of this proteoglycan on the behaviour of DCN that is seen in neonatal and young menisci, could not be excluded. Naturally, further studies about this topic are essential to clarify the role of the different proteoglycans composing the meniscal matrix and their reciprocal relation.

5. Conclusion

Decorin shows an increasing in its amount trend, moving from neonatal to adult pig menisci. Adult meniscus, in porcine species, is the only one that could be considered fully mature and functional, and, even if an increasing trend is seen, no precise phenotypical switch points are seen in the age stages considered in this study.

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3.3 POSTNATAL MORPHO-FUNCTIONAL DEVELOPMENT OF DOG'S MENISCUS.

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Paper under preparation

Abstract:

Even if menisci are recognized as essential structures for the knee joint poor information are available about their morphogenesis, in particular in dog models.

This study evaluates the morpho-functional modifications that characterize meniscal development from neonatal to adult dogs.

In this study menisci from a group of Dobermann Pinchers aged 0, 10, 30 day and 4 years were analysed. Morphological development and maturation were evaluated by SEM, polarized microscopy, immunohistochemistry and biochemical analysis. The expression of collagen type I and II was analysed to study the fibres network development. In addition, biochemical analyses with DNA; GAGs and GAGs/DNA ratio was examined in order to investigate the composition of the extracellular matrix over time.

In this study, for the first time, a bulbous aspect of the canine neonatal meniscus is described.

The relation between the forces, physiologically generated by weight-bearing and movements, and morphologic changes is also investigated matching literature described behavior and morphological findings.

The results of the current study show that meniscus born not functional and that it acquired its full task only with time and after the application of proper stresses given by physiological movements and loadings.

This study provides a better knowledge of the physiologic development of meniscus through time and could be useful to understand the behavior of this structure in the light of its tissue bio-engineering.

Key words: meniscus, development, morphology, ECM, GAGs, Collagen fibres, SEM, dogs.

1. Introduction:

Knee joints comprehend two fibrocartilaginous wedge-shaped structures: the menisci. Their role within the joint has been recognized as crucial for the conservation of knee wellness. Menisci are responsible of knee stability [as reviewed by Gabrion et al., 2005; Freutel et al., 2014; and Fox et al. 2015], congruence between femoral condyles and tibial plateau [Kettelkamp and Jacobs, 1972; Walker and Erkman, 1975; Gabrion et al., 2005; Di Giancamillo et al. 2017; Peretti et al., 2019], absorption of compressive stresses [Krause et al., 1976; Kurosawa et al., 1980; Voloshin and Wosk, 1983; Arnoczky et al., 1987; Fithian et al., 1990; Gabrion et al., 2005; Fox et al. 2015]; the conversion of compressive load into circumferential stresses [Gabrion et al., 2005; Krupkova et al. 2018]. Moreover, they are also involved in proprioception [as reviewed by Gray, 1999, Fox et al. 2015 and Gabrion et al., 2005], nociception [as reviewed by Messner and Gao, 1998; Gray, 1999, and Fox et al., 2015] and in the distribution of nutrients throughout the articular cavity [Bird and Sweet, 1987, 1988; Renstrom and Johnson, 1990; as reviewed by Fox et al. 2015]. These functions are clearly related to tissue composition. Menisci are highly hydrated tissues (water constitutes the 72% of the whole tissue) formed by a well-organized collagen network and a rich-of-proteoglycans matrix [Makris et al. 2011]. The action of proteoglycans (PGs) is explicated by the ability of their negatively-charged glycosaminoglycans terminuses to re-call and release water molecules in response to compression and, thus, generating a hydrostatic pressure that gives viscoelastic properties to the tissue [as it has been demonstrated in bovine meniscus by Mahmood, 2019]. The force generated by the compression deforms the wavy circumferential collagen fibres that in this way tend to displace transversally [as reviewed by McDermott et al 2008], however, the presence of tie-radial fibres of collagen type II disposed perpendicular and around the former, avoids excessive displacement and allows to transform this force via the so called “hoop stress” [Krupkova et al., 2018]. Furthermore, a strict structural relationship between collagen fibres (principally those of type II) and GAGs (in particular aggrecan) has been described in canine meniscus by Valiyaveettil et al. (2005).

The acme of meniscal maturation (i.e. the adult meniscus) is characterized in all the species by an almost avascular tissue (only the outer third of the meniscus remains directly vascularized) that show the double and regional-dependent features: an inner, avascular and cartilaginous-like zone (also named white-white zone), rich in proteoglycans (PGs) and collagen type II and an outer, vascular and fibrous-like zone (named red-red zone). between these two an intermediate zone, that shows midway features between the former two (aka white-red zone), is also described [Di Giancamillo et al. 2014; Di Giancamillo et al. 2017; and as reviewed by Makris et al. 2011]. Moreover, also the collagen fibres arrangement shows some degree of regionalization: collagen type I bundles are arranged circumferentially in the outer zones of meniscus and in the tibial side, to withstand tensile forces, while type II collagen disposed in radial direction, are majorly expressed in the inner zone, associated to glycosaminoglycans (GAGs), in response to compressive force [as reviewed by Fox et al., 2012].

Joint motion and the postnatal stress of weight-bearing are the principal factors that determine the phenotypical and architectural changes that characterize the maturation process of the meniscus. Different studies focused on meniscal origin and development were performed in animal models (murine and chicken) and in human embryos. These studies reported that meniscus formation happens at 8 days in chicken embryos [Mikic et al., 2000], at 15 days in mice embryos [Gamer et al. 2016] and as early as the 7th weeks of gestation in human embryos [Uhthoff and Kumagai, 1992]. While the characteristic semilunar wedge shape of mature menisci is attained in humans between the 8th and 10th week of gestation [as reviewed by Fox et al., 2012].

Mice models provided useful information about the embryologic development of meniscus. In this species menisci originate from a condensation of cells present in the interzone, the area of the future formation of the knee joint [Gamer et al., 2016; Hyde et al., 2008]. Medial and lateral menisci derive from two different cell strains of the joint embryonic gem: the cells that compose the inner zone of the medial meniscus derives from resident cells of the anlagen that momentarily switch off the expression of collagen type II (typical of the cartilaginous tissue) to form the interzone at day 13.5 of embryo's development; on the other hand, the lateral meniscus and the outer medial meniscus derive from cells that invade the developing knee joint from the area adjacent to the interzone at day 14.5 In mice at birth, the double nature of meniscus is already evident with an outer vascular zone composed of elongated fibroblasts and an avascular inner zone with round fibro-chondrocytes within a rich-of-PGs extracellular matrix. Moreover, in the superficial zone of the meniscus, adjacent to the articular cartilages of the femur and tibia, are present ovoid shaped cells in about 1–2 cells thick layers [Gamer et al. 2016]. This zone becomes smooth and intact at 1 week of age, time that coincides with the starting point of the expression of type II collagen by the inner region and the superficial zone cells [Gamer, et al., 2016]. At 8 weeks of age, the murine meniscus possesses the organized matrix and the cellular phenotypes that are typical the adult one [Gamer et al., 2016].

Similar patterns of post-natal meniscal maturation have been described in swine [Di Giancamillo et al., 2014] and ovine [Melrose et al., 2005] models and in human meniscus [as reviewed by Makris et al. 2011 and Fox et al. 2012], with each species that is characterized by the insurgence of the different developmental changes at different time points.

Few studies are focused on meniscal pre- and post-natal morphogenesis both in human [Fukuzawa et al. 2009; Koyuncu et al., 2017] and animal models [Melrose et al. 2005; Hyde et al., 2008; Di Giancamillo et al. 2014; Gamer et al., 2016].

Whatever model is chosen, it is necessary to take in mind that in any case will persist some differences between human and animals, starting from the different type of locomotion (bipedal *vs* quadrupedal) and the different time points of structure maturation: a perfect animal models for human is still lacking [Ribitsch et al. 2018; Proffen et al, 2012]. The fundamental differences between human and animals

highlight the importance of the specific knowledge about each animal model's meniscus nature and development to decrease the interspecific variability of results. Canine model is usually adopted as models for meniscal pathologies and therapies, but dogs are fundamental in meniscal research even because of their spontaneous predisposition to meniscal lesions, primary or, more often, secondary to cranial cruciate ligament rupture [Krupkova et al., 2018]. Nevertheless, information about physiological meniscal structure in dogs are very scarce, the greater amount of the information provided in this species are focusing on the therapies or on the behavior of the meniscus after cruciate ligament rupture.
To our knowledge, this is the first work that consider the physiological variations of dog's meniscus during growth and may provide valuable information for translational medicine and in the field of the development of novel tissue repair strategies.

2. Materials and Methods:

2.1. Study design

The postnatal morphogenesis of canine menisci was evaluated, focusing on collagen fibers arrangement and matrix deposition, in a population of neonatal to adult dog cadavers. Neonatal (0 day; n: 8), 10-days (n: 4), 30-days (n: 4) and 4-years old (adult, n: 3) dog's cadavers were evaluated. It was chosen, to avoid inter-breed variances, to utilize only animals belonging to the same (large) breed: Dobermann Pinscher (adult weight > 35 kg). The absence of any signs of orthopedic pathologies was the inclusion criteria for this study and was confirmed by post mortem evaluation and radiographic imaging. Each included animal died due to causes not related to the present study. The Ethic Committee of the University of Milan (OPBA, 58/2016) approved the use of these cadavers for research purpose. Each sample was harvested after capsular tissue and ligaments removal and then menisci were sectioned according to the purpose of the study as described below.

2.2. Electronic Scan Microscopy

One meniscus for each age (medial and lateral, total n: 8) were evaluated as a whole by means of Electronic Scan Microscopy (SEM) as follow: samples were immediately fixed in 2.5% glutaraldehyde in Sorensen phosphate buffer 0.1M. After several rinsing in the same phosphate buffer, they were dehydrated in a graded alcohols series, critical-point dried in a Balzers CPD 030, sputter coated with 3 nm gold in a Balzers BAL-TEC SCD 050 and examined under a Zeiss EVO LS 10 scanning electron microscope.

2.3. Morphological analyses: histology and double immunofluorescence

Menisci from each time points were sampled and fixed in buffered 10% formalin (Bio-Optica, Milan, Italy) for 24 h, dehydrated and embedded in paraffin (medial and lateral, n.12 at 0 days, n.4 at 10 and 30 days and n.4 for adults, total specimens=24). Microtome longitudinal sections (4-µm thickness) of the entire tissue (neonatal and 10-days samples) or of portioned meniscus (30-days and adult samples) were

analysed for describing both meniscal structure and GAGs deposition in the matrix. When portioned, menisci were subdivided with two transversal cuts in three sections: the anterior and posterior horns and the central body (Fig. 1).

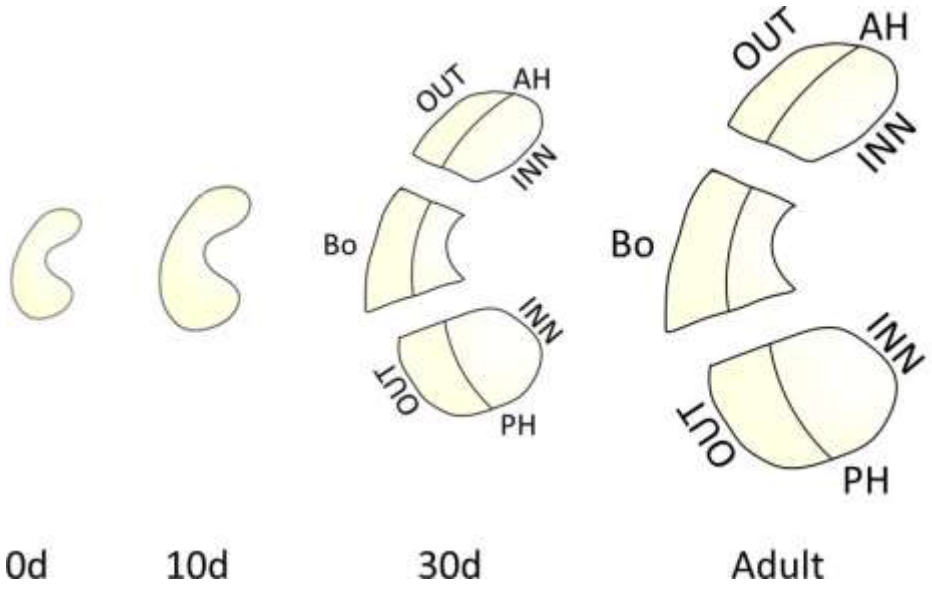

Fig. 1 Sampling procedure: 0- and 10-days menisci were analysed as a whole, 30-days and adult menisci were analysed as portions. AH: anterior horn; Bo: body; PH: posterior horn; INN: inner zone; OUT: outer zone.

Safranin-O was performed as histochemical staining to highlight the GAGs presence within the tissue. Other sections were analysed by means of polarized light microscopy after Sirius-red histochemical staining to assess the spatial orientation of the collagen networks, highlighted by the birefringence of the fibres. The samples were analysed with an Olympus BX51 light microscope (Olympus, Opera Zerbo, Milan Italy) equipped with a digital camera.

Moreover, double immunofluorescence was performed to reveal the localization and the possible co-localization of different types of collagen (type I and II). After rehydration, heat-induced antigen retrieval was performed. After washing three times in PBS (pH 7.4), sections were incubated with the first-step primary antiserum, 1:50 collagen type I (Abcam, Cam- bridge, UK) for 24 hrs at 18–20°C, then washed in PBS, and subsequently treated with the Avidin–Biotin blocking kit solution (Vector Laboratories Inc., Burlingame, CA USA). The sections were then washed in PBS for 10 min. and incubated with a solution of goat biotinylated anti-rabbit IgG (Vector Laboratories Inc.), 10 µg/ml in Tris-buffered saline (TBS) for 1 hr at 18–20°C. After rinsing twice in PBS, the sections were treated with Fluorescein–Avidin D (Vector Laboratories Inc.), 10 µg/ml in $NaHCO_3$, 0.1 M, pH 8.5, 0.15 M NaCl for 1 hr at 18–20°C. For the second step of the double immunofluorescence procedure, sections were treated in a 2% hyaluronidase solution at room temperature for 30 min. The slides were subsequently treated with 1:50 anti-collagen type II antiserum (Chondrex Inc., Redmond, WA USA). Sections were rinsed in TBS for 10 min. and incubated with

10 µg/ml goat biotinylated anti-mouse IgG (Vector Laboratories Inc.) for 1 hr at 18–20°C. The sections were then washed twice in PBS, and treated with Rhodamine–Avidin D (Vector Lab- oratories Inc.), 10 µg/ml in NaHCO3, 0.1 M, pH 8.5, with 0.15 M NaCl for 1 hr at 18–20°C. Finally, slides with tissue sections were embedded in Vectashield Mounting Medium (Vector Laboratories Inc.) and observed using a Confocal Laser Scanning Microscope (FluoView FV300; Olympus). The immunofluororeactive structures were excited using Argon/ Helio–Neon–Green lasers with excitation and barrier filters set for fluorescein and rhodamine. Images containing superimposition of fluorescence were obtained by sequentially acquiring the image slice of each laser excitation or channel. In double immunofluorescence experiment, the absence of cross-reactivity with the secondary antibody was verified by omitting the primary antibody during the first incubation step.

2.4. Biochemical analyses

Medial and lateral meniscal samples were pooled for the biochemical analysis. Whole menisci of all animals (n.18 at 0 days, n.10 at 10 and 30 days and n.6 for adults, total specimens= 42) were digested in papain (Sigma-Aldrich, Milan, Italy) for 16–24 h at 60°C: 125 mg/mL of papain in 100mM sodium phosphate, 10mM sodium EDTA (Sigma-Aldrich), 10mM cysteine hydrochloride (Sigma-Aldrich), 5mM EDTA adjusted to pH 6.5 and brought to 100mL of solution with distilled water. Later, the digested samples were assayed separately for proteoglycan and DNA contents. Proteoglycan content was estimated by quantifying the amount of sulphated glycosaminoglycans using the 1,9-dimethylmethylene blue dye binding assay (Polysciences, Inc., Warrington, PA) and a microplate reader (wavelength: 540 nm). The standard curve for the analysis was generated by using bovine trachea chondroitin sulphate A (Sigma). DNA content was evaluated with the Quant-iT Picogreen dsDNA Assay Kit (Molecular Probes, Invitrogen Carlsbad, CA) and a fluorescence microplate reader and standard fluorescein wavelengths (excitation 485 nm, emission 538 nm, cut-off 530 nm). The standard curve for the analysis was generated using the bacteriophage lambda DNA supplied with the kit.

2.5 Statistical Analysis

Biochemical results (GAGs, DNA and GAGs/DNA ratio) were analysed with 2-ways ANOVA with the whole meniscus (neonatal and 10 days old meniscus) or meniscal portions (30 days old and adult meniscus: anterior horn, body, posterior horn) and ages (0 days, 10 days, 30 days post-partum and adult) as main factors. The statistical analysis was performed using the general linear model of the SAS (version 8.1, Cary Inc., NC). The individual meniscal samples were considered to be the experimental unit of all response variables. The data were presented as least squared means ± SEM. Differences between means were considered significant at $p<0.05$.

3. Results:

3.1. Electronic Scan Microscopy

SEM imaging allows to see the different shape of dog meniscus during growth.

Neonatal meniscus is characterized by the presence of a bulgy structure (Fig. 2A) that during growth tends to flatten, firstly in the inner zone (10 days old meniscus, Fig.2B) and then even in the outer zone (30 days' meniscus, Fig.2C) until the achievement of the completely smooth adult final shape (Fig. 2D).

3.2. Morphological analyses: histochemistry and double immunofluorescence

Matrix deposition and constitution and collagen fibres' arrangement were evaluated by means of histochemical staining and double immunofluorescence.

Safranin-O staining (specific for GAGs) shows how cells morphology and matrix composition vary during growth: Neonatal meniscus is characterized by a huge number of elongated (fibroblast-like) cells (Fig. 2E, arrow) and few GAGs (Fig. 2E, asterisk), principally localized in the pericellular matrix (Fig. 2E); 10-days meniscus does not show pronounced differences compared to the previous stage, without considering the higher number of rounded cells (Fig. 2F, arrowhead) present among the elongated ones (Fig. 2F, arrow) and a higher quantity of GAGs spread all over the extracellular matrix (Fig. 2F, asterisk). The rounded shape of the nuclei is more displayed in the 30-days meniscus (Fig. 2G, arrowhead), in association with an even more abundant matrix deposition (Fig. 2F, asterisk). All these features are extremized in the adult meniscus in which cells assume a clear fibro-chondrocytic-like shape (Fig. 2H, arrowhead) and the GAGs/collagen relation is clearly visible (Fig. 2H, asterisk).

It is also possible to note the presence of some bulging in meniscal surface in both neonatal and 10-day meniscus (Fig. 2E and 2F, circles), which disappear since 30-day time points (Fig. 2G), until the definitive acquisition of a smooth and regular surface of adult menisci (Fig. 2H). Through the polarized light microscopy evaluation, it was possible to establish that these bulges are actually formed by collagen fibres that assume a yarn-like conformation (previously seen as bulges; Fig.2A) that protrude from the surface of meniscus in the first phases of development (0- and 10-day; Fig. 2I and Fig. 2J, respectively) and follow a disorganized arrangement. On the contrary, collagen bundles show an ordinate pattern (with a predominance of circumferential direction) in the latter stages of development (30-day and adult; Fig. 2K and 2L, respectively). Regarding the nature of collagen fibres, via double immunofluorescence (Fig. 3A-R), it was possible to recognized different aspect of maturation. Neonatal meniscus shows almost only collagen type I fibres (Fig. 3A-C; green) that surrounded a huge number of elongated fibroblast-like cells (Fig. 2A). Collagen type I and II co-expression starts chaotically at 10 days (Fig. 3D-F, yellow) and acquires a more evident regionalization and crimping in 30-days meniscus (Fig. 3G-L): in which it is possible to differentiate the inner zone, by the predominance of collagen type II fibres (Fig. 3K-L, red), from the outer zone, that presents a co-localization of both collagen types (Fig. 3H-I, yellow).

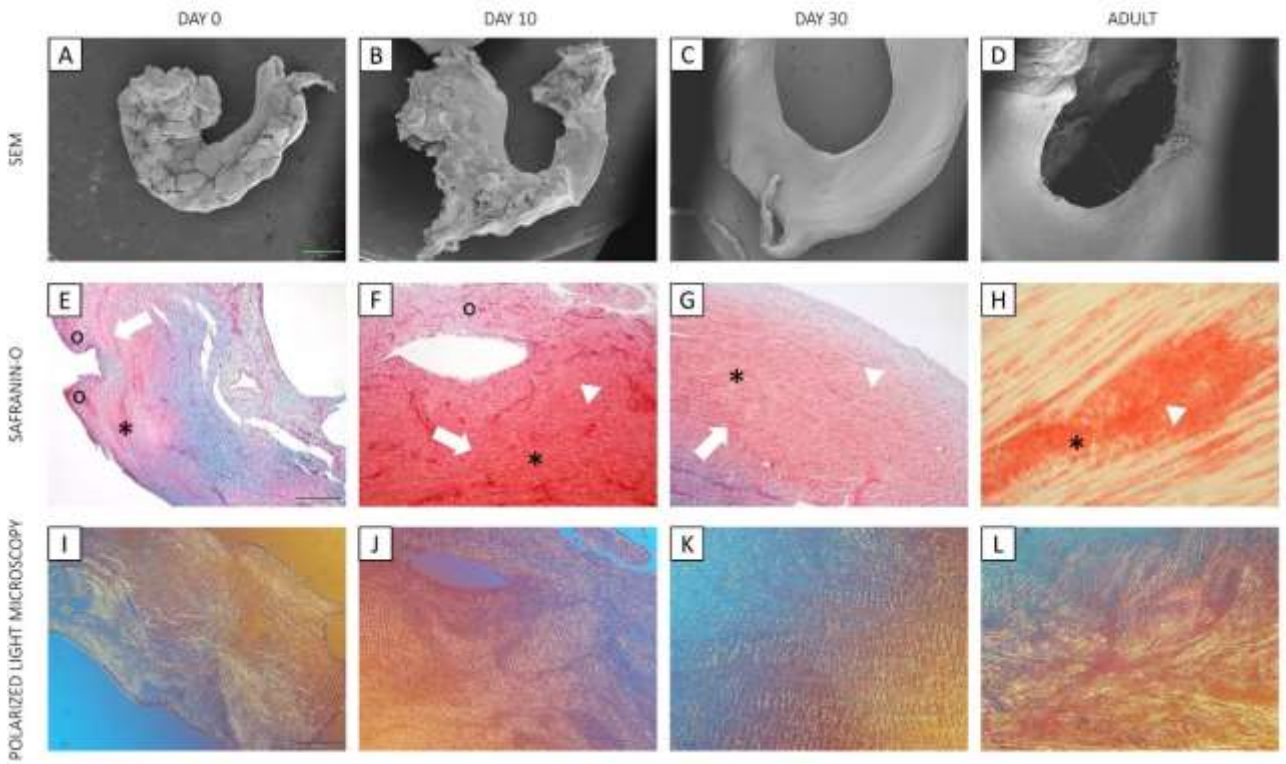

Fig. 1 SEM (A-D), Safranin-O (E-H) and Polarized light microscopy (I-L) analysis of neonatal (A; E; I), 10-days (B; F; J), 30-days (C; G; K) and adult (D; H; L) menisci. Meniscal shapes (A-D), ECM deposition (E-H; asterisks) and collagen fibres arrangement (I-L). Some bulging (E-F, circles) are present in the neonatal and 10-days menisci, mirroring what it is showed by SEM imaging (A-B). These bulging correspond to yarn-like disposed collagen fibres, that over time assume the consuetudinary woven aspect (M-N).

The same regionalization persists and is magnified in adult meniscus (Fig. 3M-O), in which the double nature of meniscus is highlighted by the presence of an outer fibrous zone (characterized by the co-expression of very crimped collagen type I and II fibres; Fig. 3N-O) and a cartilaginous-like inner zone (in which a clear chondrocytic-like phenotype may be recognized; Fig 3Q-R).

The main differences between the two latter phases are in the number of cells and the cellular phenotyping: in the adult tissue few cells (rounded, chondrocytic-like), completely surrounded by a rich matrix (Fig. 3R), are counterposed to the still numerous cells of the earlier phase, characterized by a lower (qualitative) ECM/cell ratio.

3.3. Biochemical analysis

Quantification of DNA and GAGs is essential to validate the authenticity of the suggestions provided by qualitative analysis. Moreover, they provide, through the GAGs/DNA ratio, an idea of how much the cellular phenotype of a certain time-point is maturely functional.

Biochemical analysis confirmed the suggestion that cellularity decrease over the time starting from neonatal to adult (Fig. 4A): neonatal high cellularity could be compared only with the 10-days meniscus (there is no statistically significant difference between them), while 30-days and adult present lower cellularity (with the latter that present the lowest absolute value; for both $p<0,01$). The same decreasing trend is observed in GAGs deposition (Fig. 4B). It is possible to differentiate two main clusters ($p<0,01$):

1976 the neonatal/10 days group and the 30 days/adult one. Among the latter, it is still possible to differentiate
1977 other two groups with a lower statistically significant difference ($p<0,05$).

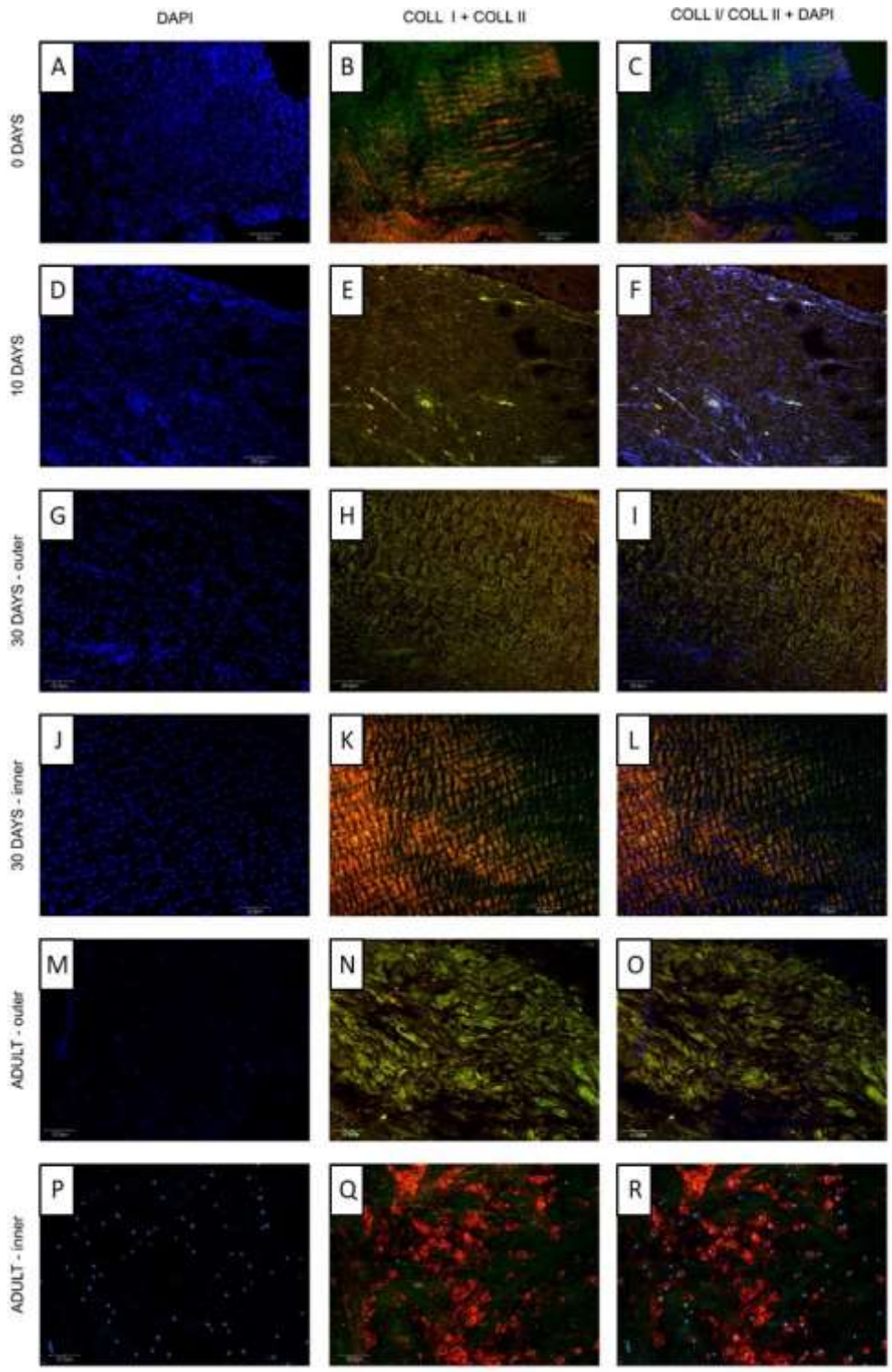

Fig. 2 Double-immuno-fluorescence of neonatal (A-C), 10-days (D-F), 30-days (G-I: inner zone; J-L: outer zone) and adult meniscus (M-O: inner zone; P-R: outer zone). Note the different grade of maturation present through each experimental point: the neonatal (B-C) and the 10-days (E-F) menisci are characterized by almost only collagen type I and numerous fusiform cells. The first signs of differentiation are showed in the 30-days meniscus in which the differentiation between the inner, rich of collagen type II, and the outer, rich of collagen type I, areas appear, however these are associated to a still fusiform nuclear morphology. This difference characterizes also the mature adult meniscus (N-O and Q-R), associated to the presence of few, and functional, cells (rounded in shape).
Collagen type I: green; Collagen type II: red; co-expression of collagen type I and II: yellow; DAPI: blue.

However, even if 30-days meniscus shows some common characteristics with the adult one, the GAGs/DNA ratios show how the latter is the only that present a mature functional tissue (FIG 4C, p<0,01) in which a small number of cells is able to produce a matrix rich of GAGs.

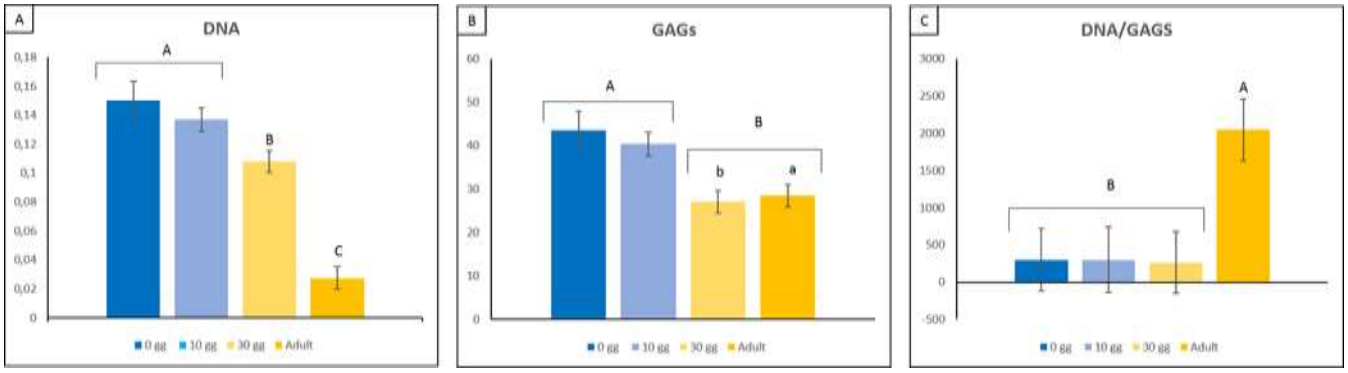

Fig. 3 Biochemical analysis. DNA (A); GAGs (B) and DNA/GAGs ratio (C) are showed. Neonatal and 10-days menisci present quite equal values for all the considered parameters (A-C). 30-days menisci show intermediate features between the neonatal-10-days pair and the adult menisci (A-C). However, adult menisci are the only that present a functional cell population (higher DNA/GAGs ratio; C).

4. Discussion:

The results of the current work stress once again the concept that meniscus born not functional and it acquired its full functionality only with time and, probably, after the application of physiological stresses given by physiological movements and loadings.

Meniscus undergoes to different structural and functional changes during its maturation process. Neonatal meniscus is characterized by a clearly immature tissue due to the association of numerous elongated fibroblast-like-cells, predominance of disorganized collagen type I fibres and poor expression of matrix components. However, this tissue achieves the physiologically expressed morpho-functional duality of meniscus throughout a series of modifications that lead to the full maturation, characterized by the presence of cells' phenotypes and collagen regionalization (fibro-chondrocytic-like cells and prevalence of collagen type II, in the inner region *vs* fibroblast-like cells and co-expression of collagen type I and II in the outer one) and by the presence of a functional ECM (elevated GAGs/DNA ratio).

These changes show their acme in the 10-30 days interval and are well explained by the evaluation of the microstructure of the meniscus which draw a picture of an immature neonatal meniscus that initially looks like a bulbous structure and gradually becomes smoother and acquires its function, till the adult well-known and functional shape. The bulgy structure of neonatal meniscus is probably due to the aggregation of clumps of disorganized collagen fibres (Fig. 2E-F and 2I-J) that are not completely stretched until the 30-days stage (Fig. 2G and Fig. 2K). The stretching of the fibres starts from the inner portion that is probably the first and the most compressed zone (Fig. 2F and 2J).

If the prime mover of the fibres' stretching is the application of biomechanical forces (body loading and full movement of the hindlimbs) or a physiological development of meniscal structure (not related to external factors) is not possible to be said without further studies focused on this topic.

However, Giorgi et al. (2014) showed through their mechanobiological 3D simulation of joint morphogenesis how the presence of biomechanical forces is more effective on the morphogenesis of joints respect to the application of growth factors.

Mechanically driven adaptive responses have been described in human bone morphogenesis and grouped together under the umbrella of the "Wolff's Law" [as reviewed by Mittag et al., 2015]. In agreement with the "form follows function" principle coined by d'Arcy Thompson [as reviewed by Mittag et al., 2015] we hypothesize that the same laws may be extend even to the morphogenesis of meniscus, even id new studies are required.

In this context, the complete achievement of the "smooth" shape of the meniscus in a period range between birth and 30 days, can be ascribed to the known behaviour of puppies during early postnatal period: initially puppies are unable to use their hindlimb, they typically crawl by dragging themselves on their venters with their front legs for all the first week [Greer, 2014]; they start using the hindquarters purposefully for propulsion around the end of the first week or at the beginning of the second week [Greer, 2014]; the complete ability to walk on the four legs is achieved between the third and the fourth weeks [Greer, 2104].

The described time ranges correspond almost perfectly to our sampling points and the milestones achieved from the puppies are congruent with the structural modifications of the meniscus described in the current study. According to this, we can suggest that at birth, because there are no biomechanical forces applied upon meniscus (day 0: no weight-bearing on the hindlimbs), the bulbous aspect is shown throughout the tissue. At 10 days postnatal (corresponding to the initial propulsion and weight-bearing) the bulbous appearance is well showed only in the outer, and less compressed, area. Finally, at 30-days the full propulsive ability is realized and so the physiological morphology of the meniscus (that is also seen in adult).

Ten-thirty days interval could be considered the starting point of the meniscus specialization and maturation accordingly to the puppies' behaviour in this time lapse, however it does not comprehend the complete achievement of the maturation, seen the incapacity of the 30-day meniscus to produce a functional matrix (qualitatively, Fig. 2G, 3F; and quantitatively, Fig. 4C).The application of gait bearing may result essential for the stretching of collagen fibers, since the physiological intrauterine movements (characterized by non-weight bearing) described in dog's foetuses [Kim and Son, 2007] seems to not be enough.

Due to the strict relationship between collagen fibres (particularly of type II) and GAGs described by Valiyaveettil et al. (2005) in dogs, and due to the fact that collagen type II is produced throughout the

meniscal tissue only in later stages of development, as it has been described in pigs [Di Giancamillo et al. 2014] and sheep [Melrose et al., 2005], one hypothesis for this findings may be that the production of a mature-like matrix in response to the applied physiological force generates the appropriate stimulus (GAGs-depending hydrostatic pressure?) to stretch and organize the collagen fibres in the proper disposition, then, once the correct arrangement of the fibres is achieved, the number of cells starts to decrease and the mature tissue is finally formed.

However, to better clarify the role of each tissue components, further studies, perhaps focused on the others ECM components (i.e. perlacan, biglycin, decorin, collagen type VI and XI), are essential.

Neonatal meniscus was studied in human being because of the presence of different congenital pathologies (such as discoid meniscus). Different shapes of meniscus were found in fetal and neonatal human meniscus (from semilunar to complete discoid type [Koyuncu et al. 2017]), however, these studies usually utilized macroscopic evaluations of this structure and no one has described the neonatal bulbous shape that is presented in this article. Furthermore, the same appearance was not described in other animals (ie. pigs [Di Giancamillo et al. 2014] or sheep [Melrose et al. 2005]) of the same age and this may indicate a specie-specific feature of dog meniscus.

However, it must be considered that the behaviour of piglet and lamb at birth is very different respect to the behaviour of the inept dog puppies. Puppies born at an earlier developmental stage, before their bodies are matured enough to walk properly, on the other hand, piglets and sheep born almost fully able in movements. This different behaviour may be anatomically reflected in an incomplete maturation of the meniscus in the former. A comparison between animals with inept-at-birth offspring (as dogs and cats) and able-at-birth offspring (as pigs, cows and horses), and an evaluation of the other structures that composed the musculoskeletal system (such as ligaments, tendons and bones) to assess if the differences seen in meniscus are present somehow also in other structures, may be of useful to fill the gaps that still exist in the knowledge of the physiological behaviour of these structures.

The knowledge of the developmental process of a structure has a capital importance to comprehend its physiologic anatomy and function and the possible behaviour of this structure under different stimuli in the attempt to replicate or replace it.

5. Conclusion:

This work highlights how dog meniscal structure changes its morphology among different age stages and the possible role of exogenous physiological stimuli in this differentiation.

The chronological congruence of meniscal structure and puppies' postnatal behaviours lead to the supposition of a crucial role of the biomechanical forces physiologically acting on meniscus in the development of its ultimate shape and functions.

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Conflict of Interest Statement

The authors confirm that there are no conflicts of interest.

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4. Exogenous factors:

4.1 SWINE MENISCUS: ARE FEMORAL-TIBIAL SURFACES PROPERLY TUNED TO BEAR THE FORCES EXERTED ON THE TISSUE?

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Abstract

Menisci are subjected to different pathologies that affected the knee proper functions and biology. Over the years, different techniques were tried to repair meniscus injury, and, when the reparation was not possible, to replace or regenerate it.

These techniques still present a lack of controlled and independent clinical studies that not allow identifying their effective failure rate.

This rate could be due to a still incomplete knowledge about meniscal biology, its composition and biomechanical properties.

The purpose of this study was to analyse the relationship between the contact forces and the meniscal structures at the level of the femoral and tibial surfaces of the meniscus to improve the knowledge about this tissue, in view of the possible application in tissue engineering, for the production of meniscal scaffolds. Swine meniscal samples were studied for morphological (Safranin-O, Sirius Red and collagen type I and II), biochemical (DNA, GAGs and GAGs/DNA ratio), CT scanning and biomechanical analyses (compression and traction tests) of femoral and tibial meniscal surfaces. Results revealed a biomechanical-dependent characterization of the meniscus.

The femoral surface is characterized by a higher quantity of GAGs and a greater amount of cells ($p<0.01$ for each analysis), with the interposition of radial and oblique fibres. These features are responsible of a higher resistance ($p<0.05$) to compressive forces like that acted on by the femoral condyles.
Oppositely, the tibial surface shows a circumferential arrangement of the fibres and a poorer GAGs presence and cellular spread ($p<0.01$); these characteristics seem to allow a higher resistance ($p<0.05$) of the tibial surface to traction forces.
Results from this work provide useful information for the design and creation of meniscal substitute and suggest that the features of the meniscus are biomechanical-dependent and that its composition and structure are dependent to the different forces that femur and tibia generate upon its surfaces.
The importance of the present study is linked to how the contact forces act on the knee meniscus in particular considering the femoral condyles and tibial plateau: these results can be useful for the tissue-engineering of meniscus, providing information about meniscal biology, its composition and biomechanical properties.

Keywords: meniscus, swine, anatomy, biomechanics

1. Introduction

The knee joint comprises the meniscus, which is a structure composed of a medial and a lateral component and both located between the corresponding femoral condyle and the tibial plateau [Kohn and Moreno, 1995]. It is a white and glossy complex tissue composed of cells specialized to produce extracellular matrix (ECM). Menisci are characterized by specific regional innervation and vascularization [Di Giancamillo et al., 2014]. Both are critical components of a healthy knee joint [Greis et al., 2002]. Moreover, meniscal tissue comprises a large network of collagen fibrils, which embed the ECM, as well as water. Collagen fibrils networks are indirectly involved in the joint biomechanics by strengthening the tissue fluid pressure under load, and directly by resisting tensile forces [Shirazi et al., 2008]. The collagen fibrils network (considering composition, structure and arrangement) spatially vary within the meniscus along the depth, because collagen is the meniscal element which can contribute to the main function of this tissue itself, i.e. loading transmission [Kaab et al., 1998].
The meniscus bears many different forces such as traction and compression. It also plays a crucial role in the transmission of load, shock absorption, lubrication and nutrition of articular cartilage [Proctor et al., 1989]. All these functions are very complex and require a specialization of the meniscus itself. Since the meniscus structure is wedge-shaped, it is very efficient in stabilizing the round-shaped femoral condyle as well as the flat tibial plate [Sweigart and Athanasiou, 2001].
The contact forces acting on the meniscus inside the knee joint have been extensively studied. It was calculated that the whole and undamaged meniscus occupies approximately 60% of the contact area

between the articular cartilage of the femoral condyles and the tibial plate, while it transmits more than 50% of the total axial load applied to the joint [Noyes and Barber-Westin, 2010].

Menisci are subjected to different pathologies (principally tears and degeneration) that can affect their fundamental functions for the knee joints. It is demonstrated that pathologies of meniscus lead to gonarthrosis, with higher impact on human welfare [Dangelmajer et al., 2017; Guo et al., 2015; Sun et al., 2015]. For these reasons, different techniques were developed during time to try to treat meniscal injuries. The first technique adopted was the meniscectomy [Guo et al., 2015], this technique contemplated the removal, initially, of the whole meniscus, was than partially renovated, trying to diminish, only to whom damaged, the entity of meniscal tissue removed. However, a lack of meniscal tissue was associated to the development of arthrosis and even a partial meniscectomy (that is currently the principal method to treat this pathologies) lead, with different grades, to the same result [Guo et al., 2015]. Then, removal was abandoned, when not necessary, in favour of reparation: different techniques of reparation were developed to treat meniscal injuries [Vaquero and Forriol, 2016], but these kinds of techniques were seen to be functional only in the cases when the lesions affected the peripheral region of meniscus (i.e. the most vascularized [Di Giancamillo et al., 2017] and inclined to regeneration [Vaquero and Forriol, 2016]).

For these reasons, in the past years, the request of new techniques, which lead to the ultimate replacement or regeneration of the meniscus, increased. The focus of these techniques is to restore the knee biomechanics, distribute the load across a larger contact area (compared to meniscectomy) and potentially delayed the onset of osteoarthritis [Dangelmajer et al., 2017].

In the last years, different techniques were developed in this direction, with scaffolds replacements and allograft transplantations [Dangelmajer et al., 2017; Guo et al., 2015; Sun et al., 2015; Vaquero and Forriol, 2016], but due to their relatively new creations, these techniques still present a lack of controlled and independent long-term clinical studies that not allow identifying their effective outcomes [Dangelmajer et al., 2017].

Nevertheless, a certain failure rate is in any case present [Dangelmajer et al., 2017]. Probably, this can be ascribed to a still incomplete knowledge about meniscal biology, its composition and biomechanical properties.

The aim of the present study was to deepen the relationship between the contact forces and the knee structures, in particular to search for the differences between the femoral condyles and tibial plateau surfaces of menisci, as a base for the ultimate creation of tissue-engineered biphasic scaffolds, which can mimic the native tissue complex, for meniscal repair or regeneration.

2. Materials and methods

2.1. Study design

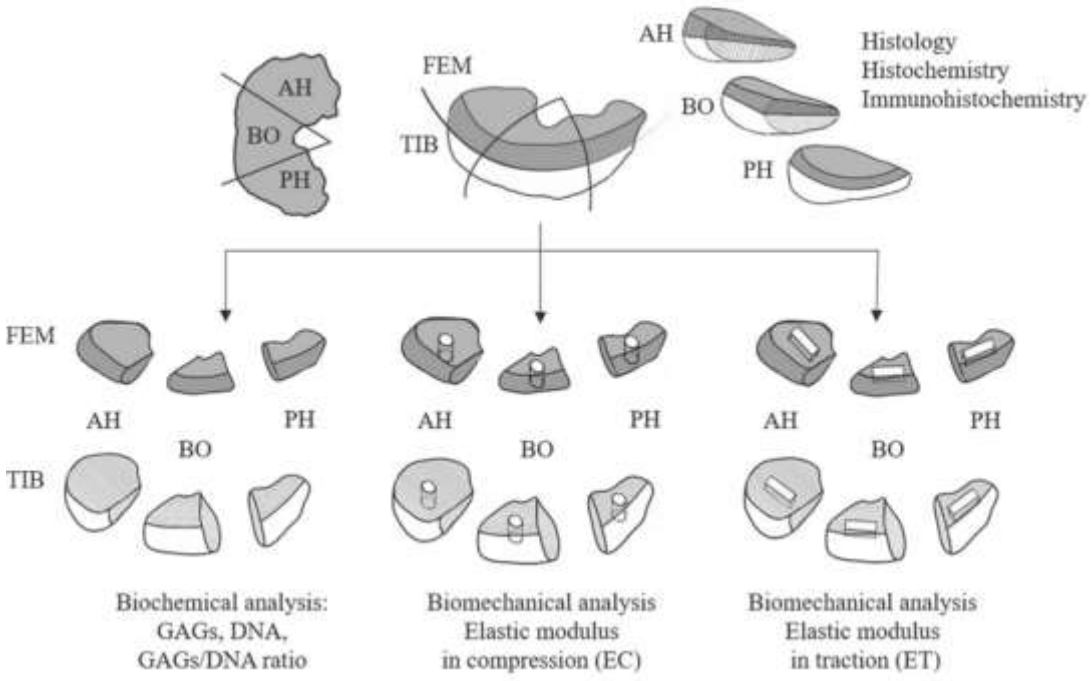
Seen the similarity with human meniscus [Proffen et al., 2012; Deponti et al., 2015] and the huge utilization as model for meniscal tissue engineering [Deponti et al., 2015] and repair [Zhang et al., 2014; Deponti et al., 2015], pig's menisci were chosen as samples for this study. The knee joints of 26 adult (~9 months old) female pigs (Landrace x Large white, average weight 75–90 kg; n=104 meniscal samples) were obtained from a local slaughterhouse and dissected to isolate the tibia and remove the menisci. Capsular tissue and ligaments were removed, and the menisci were sectioned according to the purpose of the study as described below (Fig1). Only joints presenting as healthy at dissection were included in the study. Furthermore, giving the similarities between medial and lateral menisci observed by previous micro-anatomical analysis [Di Giancamillo et al., 2014; Zhang et al., 2014], medial and lateral meniscal samples were pooled for the biochemical and biomechanical analyses. Moreover, limbs from 6 pigs were used for CT scanning as described below. The Ethic Committee of the University of Milan (OPBA, 58/2016) approved the use of cadavers for research purpose; furthermore, all the animals are dead for causes not related with the present study.

2.2 Morphological analyses: histology and immunohistochemistry

Both the medial and lateral menisci (n=28) were transversally sectioned into the three parts, corresponding to the anterior horn, central body and posterior horn (Fig. 1). Menisci were sampled and fixed in buffered 10% formalin (Bio-Optica, Milan, Italy) for 24 h, dehydrated and embedded in paraffin (total number of specimens = 84). Microtome sections (4-µm thickness) were analysed by with Safranin-O staining (SO) for describing both the menisci structure and the GAGs in the matrix as follows [Schmitz et al., 2010]. Other sections were used for Sirius Red staining [Schmitz et al., 2010] and upon the stained samples; the polarized light microscopy was performed to highlight the birefringence of the collagen fibres in order to assess the spatial orientation of the fibres networks.

Finally, sections (Fig1) were used to detect Collagen type I and II as described elsewhere [Di Giancamillo et al., 2014; Di Giancamillo et al., 2017]. Briefly, dewaxed and re-hydrated sections were treated with 5% H_2O_2 in Phosphate Buffered Saline (PBS, pH 7.4) for 10 minutes, to inhibit non-specific reactivity. Subsequently, sections were incubated with 1:50 rabbit polyclonal anti-Collagen type I (cod. NB600-408; Novus Biologicals, Littleton, CO, USA) or 1:100 mouse monoclonal anti-Collagen type II (cod. 7005; Chondrex, Redmond, WA, USA country) for 24 h at 18–20°C in humid chamber, then washed in PBS, and subsequently treated with the rabbit or mouse EnVision system, respectively, for 120 minutes at room temperature (Dakocytomation, Milan, Italy). Peroxidase activity was detected with diaminobenzidine (DAB, DakoCytomation) as the substrate. Appropriate washing with PBS was performed between each

step, and all incubations were carried out in a moist chamber. All sections were finally weakly counterstained with Mayer's haematoxylin, dehydrated, and permanently mounted.



			Portions			
Analysis performed	Menisci (tot: 104)	Sectioning	Anterior Horn (AH)	Body (BO)	Posterior Horn (PH)	Side
Histochemistry and Immunohisto-chemistry	28	Transverse sections	28	28	28	Femoral
			28	28	28	Tibial
Biochemical	20	Transverse & longitudinal sections	20	20	20	Femoral
			20	20	20	Tibial
Biomechanical (EC/ET)	50	Transverse & longitudinal sections	50	50	50	Femoral
			50	50	50	Tibial
Artro-CT	6*	Transverse & longitudinal sections	6*	6*	6*	Femoral
			6*	6*	6*	Tibial
Total	104	*not subdivided				

Fig. 1 Study design. The preparation of the meniscal samples in base of the type of technique performed is shown on the top. The table, on the bottom, resumes the number of each sample utilized for each technique and the type of cuts performed. AH: anterior horn; BO: body; PH; posterior horn. Original picture by U.P.

The specificity tests for the antibodies were verified by incubating sections with: (i) PBS instead of the specific primary antibody; (ii) PBS instead of the secondary antibodies. The results of these controls were negative (i.e. staining was abolished). The samples were analysed with an Olympus BX51 light

microscope (Olympus, Opera Zerbo, Milan Italy) equipped with a digital camera, and final magnifications were calculated.

2.3. Biochemical analyses

Sectioned menisci (Fig1, left) of all animals (n=20) were treated as described elsewhere [Di Giancamillo et al., 2017, Sosio et al., 2014; Deponti et al., 2012; Vandeweerd etal., 2011]. Briefly, the samples were digested in papain (Sigma-Aldrich, Milan, Italy) for 16–24 h at 60°C: 125 mg/mL of papain in 100mM sodium phosphate, 10mM sodium EDTA (Sigma-Aldrich), 10mM cysteine hydrochloride (Sigma-Aldrich), 5mM EDTA adjusted to pH 6.5 and brought to 100mL of solution with distilled water. Later, the digested samples were assayed separately for proteoglycan and DNA contents. Proteoglycan content was estimated by quantifying the amount of sulphated glycosaminoglycans using the 1,9-dimethylmethylene blue dye binding assay (Polysciences, Inc., Warrington, PA) and a microplate reader (wavelength: 540 nm). The standard curve for the analysis was generated by using bovine trachea chondroitin sulphate A (Sigma). DNA content was evaluated with the Quant-iT Picogreen dsDNA Assay Kit (Molecular Probes, Invitrogen Carlsbad, CA) and a fluorescence microplate reader and standard fluorescein wavelengths (excitation 485 nm, emission 538 nm, cut-off 530 nm). The standard curve for the analysis was generated using the bacteriophage lambda DNA supplied with the kit.

2.4. CT examination

Six hind limbs harvested from pig cadaver were evaluated. Medio-lateral and caudo-cranial radiographs of each stifle were performed to exclude abnormal findings. Each limb was positioned foot-first towards the gantry mimicking dorsal recumbence, with the caudal surface of the limb apposed to the CT couch. Images were acquired with a 16-slices CT scanner (Ge Brightspeed, GE Healthcare – Italy), using a bone algorithm. Scanning parameters were set as follows: kV=120, mA=230, slice thickness=0.625 mm, pitch=0.5625. Each limb was initially positioned in a neutral position. Transverse pre-arthrography CT images were acquired from 2 cm proximal to the patella to 2 cm distal to the tibial tuberosity. Twenty-five ml of iomeprol 150 mg/ml (Iomeron 150, Bracco – Italy) were injected through a 20-gauge hypodermic needle inserted into the stifle joint medial to the mid-point of the patellar tendon [adaptation of the paraligamentous technique described in the ovine stifles, Vandeweerd et al., 2012]. The joint was repeatedly flexed and extended to ensure adequate distribution of contrast medium. The limb was then repositioned on the CT couch and the CT acquisition protocol was repeated. After the acquisition in neutral position, further arthrographic images were acquired with the limb in flexion and in extension. Each limb was flexed and extended as much as manually achievable and secured to the CT couch with large velcro bands (extended: 140±2°; neutral: 116±2°; flexed: 54±4°). The same imaging protocol was then repeated. Images were reviewed by a single radiologist in order to verify meniscal movements, the

contact between femoral/tibial condyles and meniscal surfaces and meniscal compression with different positioning, focusing on the lateral meniscus. Meniscal thickness was measured at the level of the anterior and posterior horn on a MPR (multiplanar reformation) sagittal image passing through the midpoint of the femoral condyle, at a distance of 1 mm from its cranial and caudal surface respectively (Supplementary file1A: red and pink lines). Body portion thickness was evaluated in the same point, as the measure of the midpoint of the meniscus (Supplementary file1A: yellow lines). On the same image, the lengths of the contact surface between the femoral surface of the meniscus and the femoral condyle and between the tibial surface of the meniscus and the tibial condyle were also measured (Supplementary file1B: dark and light blue lines). Finally, the total length of the meniscus was evaluated (Supplementary file1C: green line). All measures were repeated three times for each positioning (extension, neutral, flexion).

2.5. Biomechanical testing

Menisci from adult swine (n=50) were stored in saline solution NaCl 0.9% and frozen at -80°C until the time of testing. At least 24 hours before test execution, samples were taken to a temperature of -24°C and then completely thawed at room temperature (23°C). Each meniscus was prepared and cut along the longitudinal axis, in its half height, in order to obtain a meniscal femoral surface and a meniscal tibial surface, too (Fig1). Compression and circumferential traction tests were performed using EnduraTEC ELF® 3200 machine, equipped with a 220 or 22N load cell depending on the test and sample.

For compression tests, 25 menisci were used. Specimens were obtained dividing each meniscal surface in the three portions after the longitudinal cut (Fig1): anterior horn, body and posterior horn (n=75). Subsequently, for each zone, a cylindrical sample perpendicular to the femoral and tibial surfaces was cut using a die cutter (Fig1, in the middle). Before testing, dimensional measurements on the specimens were made with a digital calliper (Mitutoyo Corp, Kanogawa, Japan, number of series 06,081,911, accuracy class 1). The samples were inserted into a Plexiglas cell and PBS solution was added into the cell to avoid dehydration of the specimen during the test. The samples thickness was measured from the position of the testing machine actuator, after imposing a preload of 0.1N. The sample was then subjected to a multi-ramp stress relaxation test, made of five increasing 4% strains at a velocity of 0.1%/s, followed by stress relaxation to equilibrium for 600s. The compressive modulus, Ec, was obtained for each ramp from the equilibrium data as the ratio between values of relaxation stress and the corresponding values of strain.

For circumferential tension test (n=25 menisci), we obtained representative fragments of the meniscus anterior horn, body and posterior horn on both femoral and tibial surfaces (n=75). These fragments were cut out using a scalpel, trying to obtain samples with a shape as similar as possible to a parallelepiped (Fig1, on the right) and following the circumferential tensile force direction. Before testing, dimensional measurements on the specimens were made with the same digital callipers previously indicated. Width and thickness of each sample were obtained using the callipers, length instead was measured on the

mounted specimen, considering as length the distance between the two grips after a preload of 0.1N application. The specimens were subjected to a multi-ramp stress-relaxation test, made of four increasing 4% strains at a velocity of 0.1%/s, followed by stress relaxation to equilibrium for 1200 s. A custom made chamber filled with PBS was used to keep the samples hydrated during the test.

2.6. Statistical Analysis

Statistical analyses of the biochemical (GAGs, DNA and GAGs/DNA ratio), biomechanical results (compression and traction tests) and CT measures (horns' thickness, body thickness, meniscal length, and femoral and tibial contact surfaces) were analysed with 2-ways ANOVA with surfaces (femoral and tibial surfaces) and meniscal portions (anterior horn, body, posterior horn) as main factors. The statistical analysis was performed using the general linear model of the SAS (version 8.1, Cary Inc., NC). The individual meniscal samples were considered to be the experimental unit of all response variables. The data were presented as least squared means ± SEM. Differences between means were considered significant at $p<0.05$.

3. Results & Discussion

3.1. Morphological analyses: histochemistry and immunohistochemistry

Considering the similarities of the medial and lateral meniscus already described in a previous study of our research group [Di Giancamillo et al., 2014] only data regarding morphology of the lateral meniscus are presented. Safranin-O histochemistry assessed that the amount of matrix (proteoglycans) was more evident in the inner part both in anterior and posterior horns (Fig. 2A, 2B respectively; asterisks), similarly to the results of previous works, that showed the same aspect in the inner part of ovine meniscus [Melrose et al., 2005].

Proteoglycans were also predominant within the anterior horn (Fig. 2A1, 2A3; asterisks) when compared with the posterior one (Fig2: B1, B3; asterisks), and in the femoral meniscal surfaces (Fig. 2A1, 2B1; asterisks) when compared with the corresponding tibial surfaces (Fig 2A3, 2B3; asterisks). Body portion seem to have poorer in GAGs quantity, with a very pale staining in the middle of the tissue (Fig. 2C; asterisk).

These results may suggest a role in the development of the ECM of dynamic compression forces, typically acting on the femoral surface of the meniscus. Moreover, these results suggest, accordingly with a previous work [Di Giancamillo et al., 2014], an earlier development of the meniscal anterior horn with respect to the posterior one. Sirius red stained samples were evaluated to enhance the arrangement of collagen fibres in the central point of the meniscus.

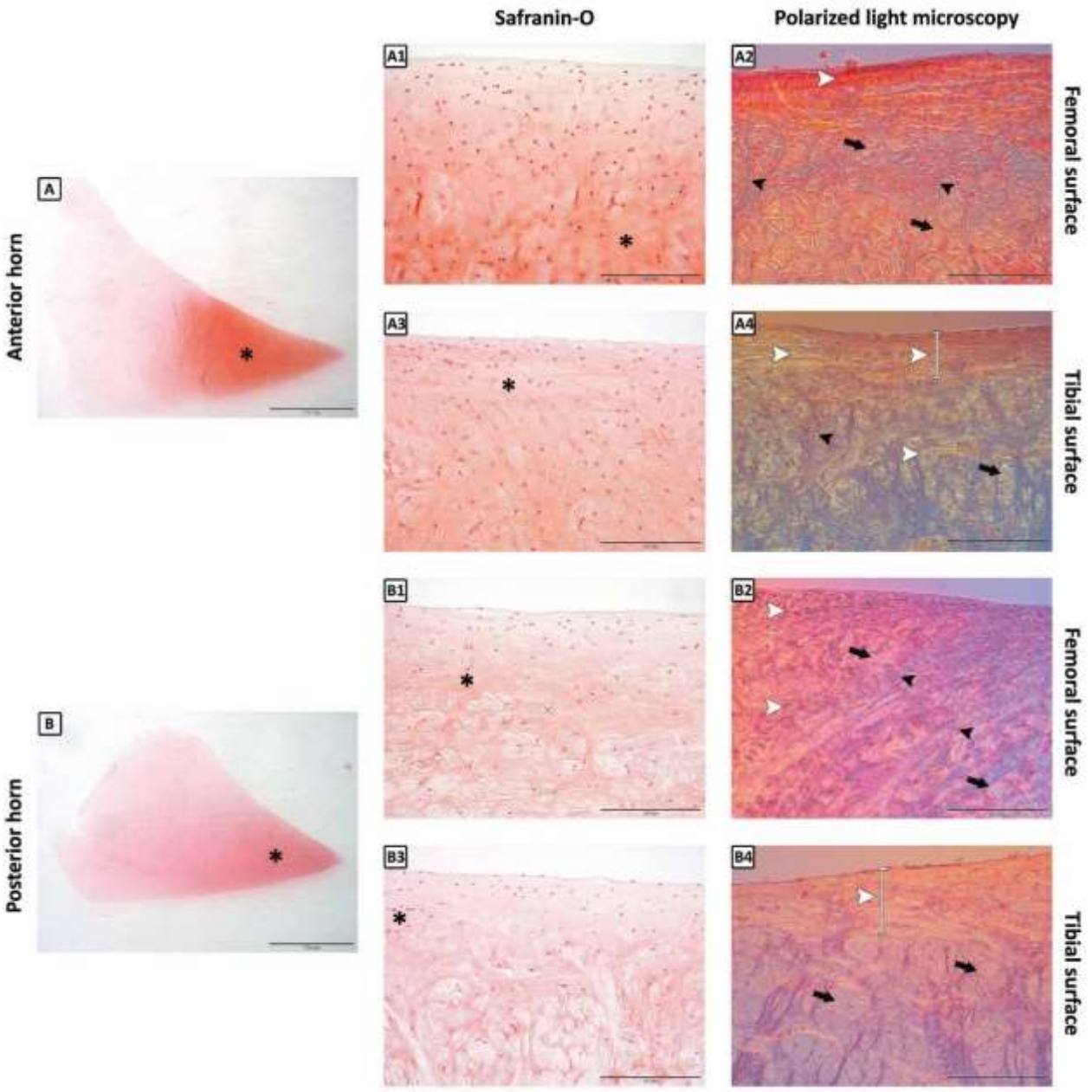


Fig 2. Histochemical findings of the lateral meniscus (Safranin O staining). (A) Anterior horn; (A1) femoral surface; (A3) tibial surface. Body (B): (B1) femoral surface, (B3) tibial surface. Posterior horn (C): (C1) femoral surface; (C3) tibial surface. Histochemical findings of the meniscus (Sirius red staining – polarized light microscopy). Anterior horn: (A2) femoral surface; (A4) tibial surface. Body: (B2) femoral surface, (B4) tibial surface. Posterior horn: (C2) femoral surface; (C4) tibial surface. A, B, Scale bar 5000 µm; A1-A4; B1-B4, Scale bar 200 µm. Black arrows: circumferential fibres; white arrowheads: radial fibres; black arrowheads: oblique fibres; asterisk: matrix deposition.

The spatial disposition of the fibres varied considering the different meniscal portions and regions. The femoral and tibial surfaces of anterior horn presented principally a radial pattern of the fibres in the superficial region, the one closer to the femoral condyle or to the tibial plateau (Fig. 2A2, 2A4; white arrowhead), as seen by other Authors in ovine, human and bovine respectively [AufderHeide and Athanasiou, 2004; Deponti et al., 2012; Vandeweerd et al., 2012; Sosio et al., 2015].

The main differences observed in this study, between femoral and tibial surfaces of the anterior horn were related to a higher incidence of oblique (Fig. 2A2; black arrowheads) and circumferential (Fig. 2A2;

black arrows) fibres in the femoral surfaces respect to the tibial ones, while the opposite trend was observed for the radial fibres (Fig. 2A4; white arrowheads). These kinds of fibres recall those observed in the study of Andrews [Andrews et al., 2013] and denominated braided organized type, present in the superficial layer and the woven organized type, present in the deeper layer.

In contrast, the same study (performed upon the anterior horn of bovine medial meniscus) showed no differences between 3D fibres arrangement in the femoral and the tibial surfaces [Andrews et al., 2013].

The posterior horn showed much different patterns between the femoral and tibial surfaces and between the deep and superficial regions of both surfaces. In the superficial femoral surface (the one closer to the femoral condyle), it was possible to recognized two layers of fibres disposed in a radial arrangement (the most superficial one, Fig. 2B2; white arrowhead) and a circumferential arrangement (deeper with respect to the previous, Fig. 2B2; black arrows), while in the deepest region of the femoral surface, there was a wider occurrence of radial (Fig. 2B2; white arrowhead), oblique (Fig. 2B2; black arrowheads) and circumferential fibres (Fig. 2B2; black arrow).

Moreover, the tibial surface of the posterior horn presented a wider radial disposition of the fibres in the superficial region (the ones closer to the tibial plateau, Fig. 2B4; white arrowhead), while in the deepest region there are fibres mostly arranged in a circumferential pattern (Fig. 2B4; black arrow).

Generally, in the posterior horn, femoral and tibial surfaces showed a conventional fibre arrangement, as already described by other works, the differences seen in this paper between the two surfaces differed from those described by those Authors for ovine and bovine menisci, respectively [Deponti et al., 2012, Vandeweerd et al., 2012] but could be grossly assimilate to those reported by AufderHeide and Athanasiou [AufderHeide and Athanasiou, 2004], and Petersen and Tilmann [Petersen and Tilmann, 1998], in human.

Body portion seem to have an intermediate pattern of fibres, with a higher prevalence of circumferential fibres (Fig. 2B2, black arrows) in the femoral surface and a higher disposition in radial (Fig2B4, white arrowhead) direction in the tibial one.

Immunohistochemistry showed a regional dependent matrix deposition: collagen I was present principally in the posterior horn (Fig. 3E1, 3E2, 3E3), in the femoral (Fig. 3E1) and tibial (Fig. 3E2) surfaces of the meniscus and in the inner region (Fig. 3E3). In the anterior horn collagen I was shown only by a nuclear staining (Fig 3A1, 3A2, 3A3; brown in the pictures). Collagen II was present in the anterior horn (for the inner, femoral and tibial parts, Fig. 3B1, 3B2, 3B3) and in the posterior horn (almost only in the inner part, Fig. 3F3). These results agreed with previous work that showed an age-dependent localization of the collagen disposition in ovine and swine [Deponti et al., 2012 and Di Giancamillo et al., 2014, respectively].

Body shows once again an intermediate pattern, with a poor, almost only nuclear, expression of collagen I (as seen in anterior horn, Fig. 3C1, 3C2, 3C3) and a poor expression of collagen II (as seen in posterior

horn, Fig. 3D1, 3D2, 3D3), but a higher presence of collagen II in the inner part respect to the latter (Fig. 3D3, asterisk).

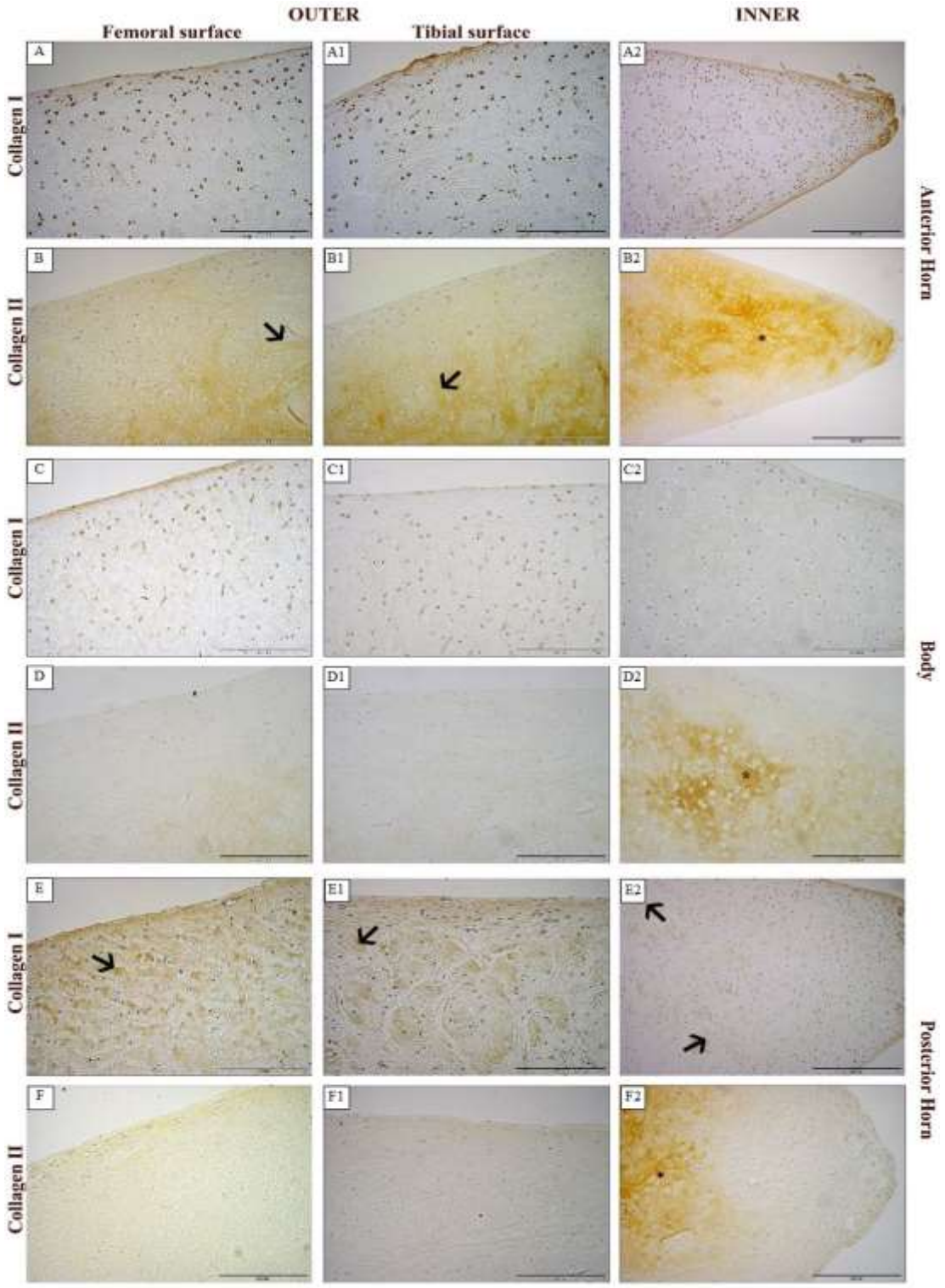


Fig. 3 Immunohistochemistry for collagen type I of the lateral meniscus: Anterior horn: (A1) Femoral surface; (A2) tibial surface; (A3) inner part. Body: (C1) Femoral surface; (C2) tibial surface; (C3) inner part. Posterior horn: (E1) Femoral surface; (E2) tibial surface; (E3) inner part. Immunohistochemistry for collagen type II. Anterior horn: (B1) Femoral surface; (B2) tibial surface; (B3) inner part. Body: (D1) Femoral surface; (D2) tibial surface; (D3) inner part. Posterior horn: (F1) Femoral surface; (F2) tibial surface; (F3) inner part. Arrows: fibers; asterisk: matrix deposition. All the figures have the same scale bar: 100 µm.

Collagen I, much more abundant in immature meniscal tissue [Di Giancamillo et al., 2014] and tendons [AufderHeide and Athanasiou, 2004], was mainly expressed by the posterior horn (Fig. 3E1, 3E2, 3E3) that, as seen previously [Di Giancamillo et al., 2014] developed later with respect to the anterior one. On the other hand, collagen II, much more abundant in mature meniscal tissue [Di Giancamillo et al., 2014] and cartilage [AufderHeide and Athanasiou, 2004] was expressed in the anterior horn (Fig. 3B1, 3B2, 3B3) which developed earlier in the meniscal tissue. Note that only the inner, and, much compressed, part of the posterior horn expressed collagen II in its matrix (Fig. 3F3). Once again, these results showed how biomechanical forces are essential for the right development of the meniscal structure.

3.2. Biochemical analysis

Biochemical analysis quantified the cellular spread and the GAGs amount through the meniscal tissue. Both DNA and GAGs contents were higher in the femoral surface ($p<0.05$) when compared with the tibial one (Fig. 4A, 4B), while the GAGs/DNA ratio showed no significant differences, but a higher value for the femoral surface (Fig. 4C).

In particular, DNA content was higher in both anterior and posterior horns of the femoral surface when compared with the corresponding tibial surface portions (Fig. 4A; $p<0.01$ and $p<0.05$, respectively). Body portions of both femoral and tibial surfaces presented no statistically significant differences (Fig. 4A).

A similar trend was present in GAGs quantification, that is characterized by a higher value in both anterior and posterior horns of the femoral side when compared with the corresponding tibial portions (Fig 4B; $p<0.01$). GAGs quantification showed also a poorer ($p<0.05$) statistically significant difference between the femoral and tibial body portion, with a higher value present in the femoral surface (Fig 4B).

GAGs were evaluated to analyse matrix content in relation with the response to compressive forces as observed by Bursac et al. (2009) in human and by Abdelgaied et al. (2015) in pigs.

The GAGs/DNA ratio showed no significant differences among all portions (Fig4F). This may be due to the fact that adult meniscus had reached a complete development, and so, cells, matrix and collagen network, achieved an equilibrate connection throughout the entire tissue, differently from what could be seen in growing menisci [Abdelgaied et al., 2015].

Biochemical analysis reiterated, once again, the presence of two well-differentiated portions: a femoral (rich of cells and glycosaminoglycans) and a tibial one (characterized by the greater relative amount of fibres). The obtained data were in agreement with previous description of the meniscus as a tissue having an inner proteoglycan rich matrix [AufderHeide and Athanasiou, 2004, 31] that resembles hyaline cartilage and an external fibrous region, more similar to ligament and tendons [Moon et al., 1984; Cheung, 1987; AufderHeide and Athanasiou, 2004; Son et al., 2012].

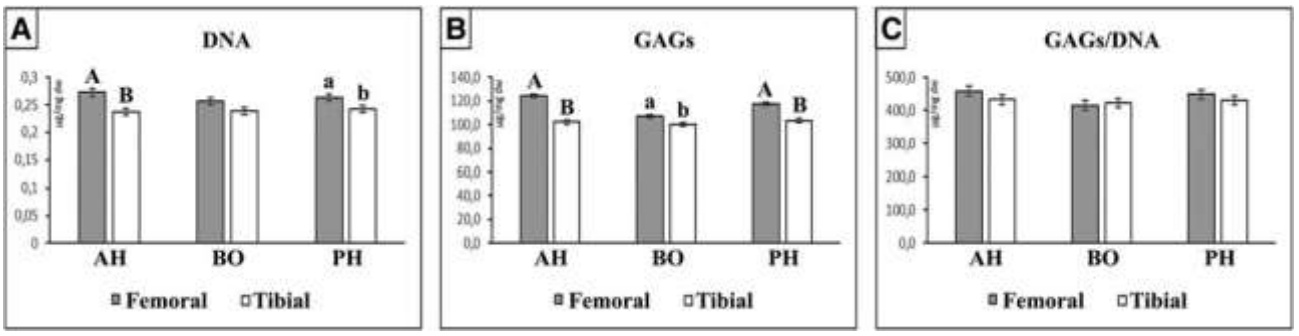


Fig 4 Biochemical analysis results. DNA, GAGs and GAGs/DNA ratio (A, B and C) are shown. DNA values (i.e. cellularity, A) and GAGs quantification (B) are higher among the femoral meniscal contact surface when compared with the tibial one. Values with different subscripts (a, b) for $p< 0.05$ and (A, B) for $p< 0.01$.

3.3. CT scanning

The results of CT analysis (Fig. 5) provided a hint of meniscal behaviour during knee's movement. During standing and movement, neutral positioning corresponded to full loading, while flexion corresponded to general stifle unloading [Fuss, 1991]. The thickness of the meniscus in different positioning seemed to suggest that compressive forces on the anterior horn, body and posterior horns (Fig. 5D) have no effects upon their macroscopic structure. The same happened for the total length of the meniscus (Fig. 5E), as their complex structure allows them to hold their shape under different stimuli. Flexion seemed to induce a noticeable reduction in the length of the contact surface among the meniscus and both the bone surfaces (for both, $p<0,01$; Figs. 5A-C, 5F and 5G). However, the femoral contact surfaces seemed to be less affected by the extension (Fig. 5F; $p<0,05$) and the tibial contact surface seemed to be not affected at all (not statistically significant respect to the neutral position, Fig. 5G). A large amount of contrast medium seemed to fill the space between the anterior horn of the meniscus and the femoral and tibial condyles (femur > tibia), suggesting a fan-like separation between the horn and the bone surfaces during flexion (Fig. 5C; red arrows). Moreover, caudal displacement of the meniscus was visible, which seemed to slip caudally along the tibial condyle (Fig. 5C, yellow arrow) as it was described by other Authors [Fowlie et al., 2011 and Masouros et al., 2010].

The observed differences were related to the decreasing contact between the cranial horn of the meniscus and the two joint heads, while the contact of the caudal horn seemed to be less influenced by the joint movement (Fig. 5A-C).

3.4. Biomechanical tests

Results of biomechanical tests showed a higher compression Elastic modulus (Ec) in the femoral surface with respect to the tibial one (Fig 6A). In particular, anterior and posterior horns presented higher Ec moduli in the femoral surfaces respect to tibial one (Fig 6A; $p<0.05$) while no differences were recorded for the bodies.

The influence of GAGs on tissue compressive force response has been previously demonstrated by other Authors both in human and pigs [respectively Bursac et al., 2009 and Abdelgaied et al., 2015].

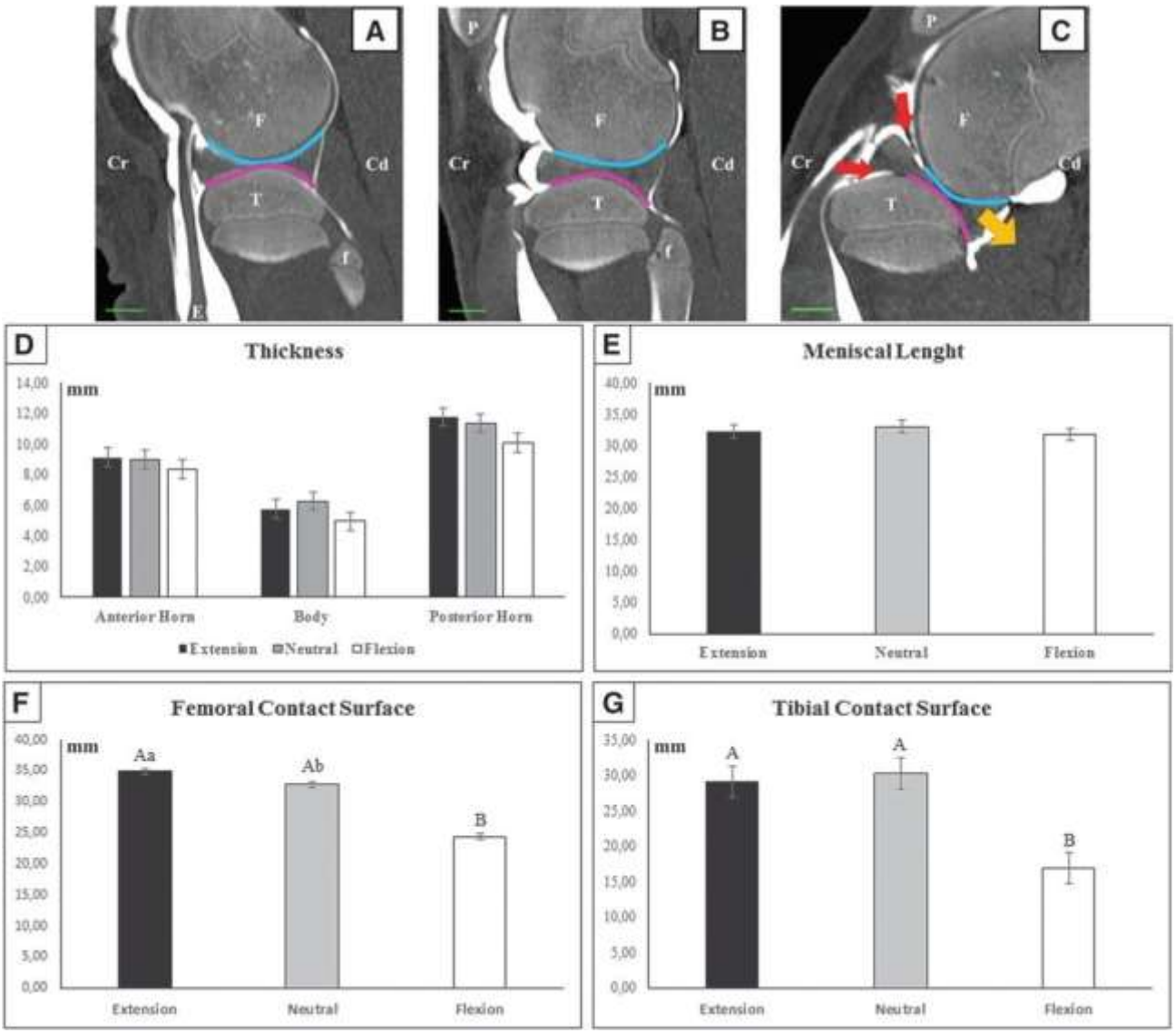


Fig. 5 CT images analysis. Representative images of meniscal changes with different positioning (lateral meniscus): A) in extension, B) neutral, C) in flexion. CT images analysis shows no statistically significant differences both for body and horns' thickness (D) and meniscal length (E) while a reduction in bone/meniscus contact surfaces is evident in flexion (F and G). Contrast mean is noticed between the meniscus and the femoral and tibial surfaces (red arrows), suggesting a fan-like separation of the cranial part of the meniscus from the bones. Caudal displacement of the meniscus along the tibial condyle is also evident (emphasized by the yellow arrow). Values with different subscripts (a, b) for $p<0,05$ and (A, B) for $p<0,01$. Scale bar=1 cm.

In contrast with our results, Proctor et al. (1989), did not find any differences in unconfined elastic modulus in compression, moving from femoral superficial to deeper regions in bovine medial meniscus [Lai and Levenston, 2010]. A higher Ec modulus in the anterior horn (at 12% strain) with respect to the posterior one, was reported by Chia and Hull (2008), in human meniscus. Unfortunately, both these studies did not consider the differences between femoral and tibial surfaces. On the other hand, it was possible to observe opposite results for what concerned the traction elastic modulus (Et) with a higher value for the tibial surface respect to the femoral one (Fig 6B). It is well known that the Et property is dependent on collagen [Abraham et al., 2011]. In particular, all the three regions (anterior horns, posterior horns and bodies) presented higher values of Et in the tibial surfaces with respect to the femoral ones (Fig

6B; p<0,05 for all comparisons). These results showed a different biomechanical behaviour in the different regions and surfaces of the meniscus related to the type of forces the act upon them.

Tensile properties of the meniscus are related with the collagen fibres presence and disposition [Abdelgaied et al., 2015]. A higher tensile modulus value of the anterior horn was also reported by other studies [Kalhon et al., 2015] in porcine meniscus. However, in a study, performed on bovine meniscus, the highest value of tensile modulus was measured in the posterior horn [Proctor et al., 1989; Abraham et al., 2011]. Therefore, these differences may be due simply to interspecific features, but their importance has to be better evaluated in the future.

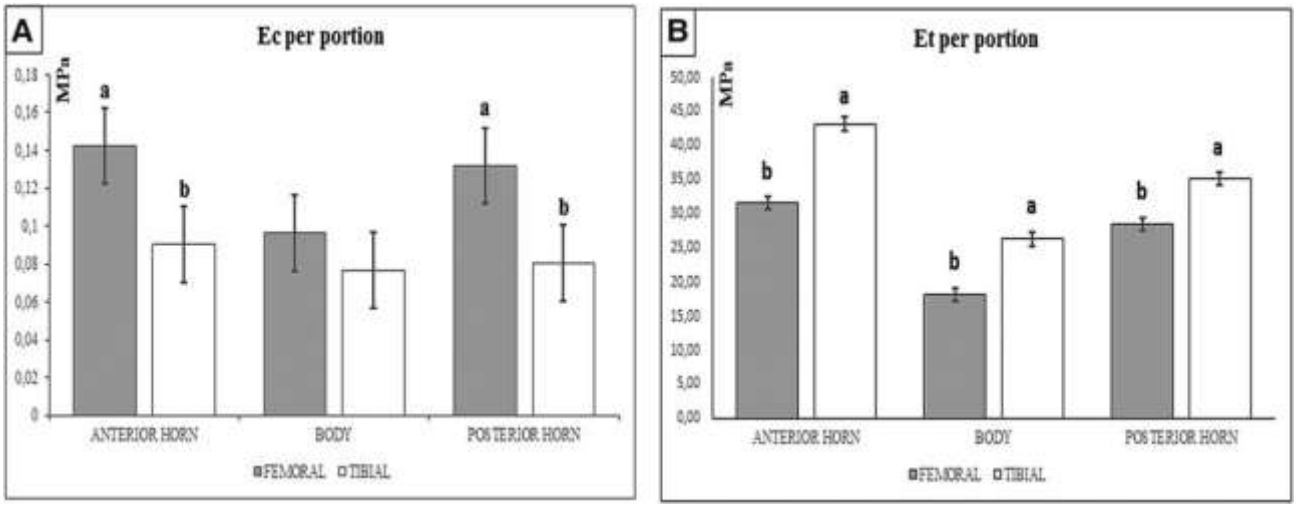


Fig 6 Biomechanical results. Compression test results (A) and Circumferential traction test results (B). Femoral meniscal contact surface shows a higher elastic modulus in compression (Ec; A) while tibial meniscal contact surface shows a higher elastic modulus in traction (Et; B). Values with different superscripts (a, b) for $p < 0.05$.

Biomechanical results draw a picture that characterized the femoral surfaces like more subjected to compressive forces (as previously suspected based on the GAGs presence enhanced by the safranin-O staining and GAGs biochemical quantification), while the tibial surfaces, in particular that of the anterior horn, like more subjected to traction forces. In fact, during physiological loading, tensile, compressive, and shear forces are generated. Consequently, vertical and horizontal forces result from the femur compressive action on the curved superior surface of the meniscus. From the horizontal force originated a radial reaction force: when the femur compresses the meniscus, it tends to deform radially, but, because of its anchorages to the tibial plateau, it generates a tensile hoop stress resulting from the radial deformation [Athanasiou and Sanchez-Adams, 2009; Muir et al., 2017].

The role of biomechanical forces is essential in the morpho-functional development of the synovial joints components and in particular, for the meniscus, in fact, it was demonstrated that the immobilization of the embryo's hind limb causes the degeneration and the disappearance of this structure [Mikic et al., 2000].

4. Conclusion

Morphological, biochemical, CT imaging and biomechanical analysis highlight the presence of two well-differentiated meniscal components: a femoral (rich of cells and glycosaminoglycans) and a tibial one (characterized by a greater amount of circumferential fibres). Their different compositions reflect their natural functions: femoral surface act as the springs of a car in response to the transmission of the compressive weight-bearing loads, consequently the tibial surface acts to dissipate the compressive forces, through the collagen fibres network with a hoop-stress mechanism, comparable to car dampers. These functions are biomechanical-dependent and linked to the fibres organization: compressive forces, due to the femoral condyles compression, sliding and rolling, induce a higher deposition of ECM, as well as a circumferential arrangement of the fibres associated with the interposition of some radial and oblique fibres in the corresponding surface of the meniscus. Moreover, traction forces are highly active on the tibial surfaces of the meniscus inducing a circumferential deposition of fibres, which are highly resistant to traction.

Results from this work, even if preliminary, provide useful information for the design and creation of meniscal substitute and suggest that the features of the meniscus are biomechanical-dependent and that its composition and structure are dependent to the different forces that femur and tibia generate upon its surfaces.

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Conflict of interest

Authors declare that they do not have any conflict of interest for the present study.

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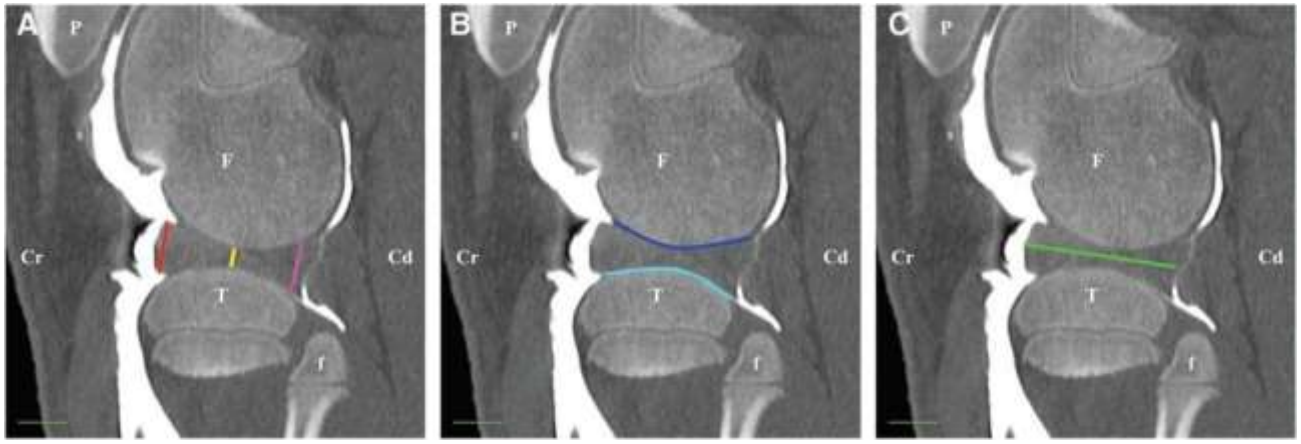

Supplementary file 1 CT measures (lateral meniscus). A. Meniscal thickness at the level of the anterior horn (red line), body (yellow line) and posterior horn (pink line). B. Length of the contact area between the femoral surface of the meniscus and the femoral condyle (dark blue line) and between the tibial surface of the meniscus and the tibial condyle (light blue line). C. Total meniscal length (green line). P=patella; F=lateral femoral condyle; T=lateral tibial condyle; f=fibula; Cr=cranial; Cd=caudal.
MPR image reconstructed on a sagittal plane passing through the midpoint of the lateral femoral condyle (neutral positioning). Bar=1 cm.

4.2 EFFECTS OF CONTINUE COMPRESSIVE FORCES UPON MENISCAL MATRIX IN A POPULATION OF 12 DOBERMANN PINSCHERS AFFECTED BY QUADRICEPS CONTRACTURE SYNDROME.

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Paper under preparation

Abstract:

Joint motion and postnatal stress of weight-bearing are the principal factors that determine the phenotypical and architectural changes that characterize the maturation process of meniscus.

In this study the effect of compressive forces on meniscus will be evaluated in a litter of 12 Dobermann Pinchers, of approximately 2 months, affected by quadriceps contracture syndrome; to investigate the effect of this kind of stimulus focusing on meniscal cells maturation and collagen fibres arrangement.

The affecting legs of this population were considered like models of continue compression while the physiologic loaded legs were considered like controls.

The results of this study suggest that a continue compressive force, applied to the native meniscal cells, triggers an early maturation of the cellular phenotype, at the expense of the proper organization of collagen fibres.

Nevertheless, an application of a compressive force could be useful in meniscal tissue bio-engineering to induce a quicker achievement of the mature cellular phenotype, and consequently the earlier production of the fundamental ECM, in order to improve cellular viability and adhesion of the cells within a hypothetical synthetic scaffold.

Keywords: meniscus, maturation, GAGs, compression, EC, Young's modulus, dog

1. Introduction

Menisci are C-shaped fibrocartilaginous wedge structures with a crucial and well-documented role in the knee joint. Load transmission across the joint [Fairbank, 1948; Arnoczky et al., 1987; as reviewed by Fox et al. 2015], shock absorption [Voloshin and Wosk, 1983; Arnoczky et al., 1987; Fithian et al., 1990; as reviewed by Fox et al. 2015], nutrient distribution[Bird and Sweet, 1987, 1988; Renstrom and Johnson, 1990; as reviewed by Fox et al. 2015], joint lubrication [Renstrom and Johnson, 1990; as reviewed by Fox et al. 2015], improved congruency [Kettelkamp and Jacobs, 1972; Walker and Erkman, 1975; Di Giancamillo et al. 2017; Peretti et al., 2019], proprioception [Wilson et al., 1969; Zimny et al., 1988; Assimakopoulos et al., 1992; Jerosch et al., 1996; Messner and Gao, 1998; Gray, 1999; Akgun et al., 2008; Karahan et al., 2010; as reviewed by Fox et al. 2015,] and nociception [Messner and Gao, 1998; Gray, 1999; as reviewed by Fox et al., 2015] are recognised as the main functions of meniscus within the knee joint.

These functions derive from the peculiar structure and composition of this tissue.

Menisci may be grossly divided, transversally, in an anterior/cranial horn, a central body and a posterior/caudal horn [Di Giancamillo et al. 2017], and longitudinally, in others three different zones: the *red-red zone*, fully vascular and strictly in contact with the joint capsule, corresponding to outer, thicker and rounded border of this wedge-shaped structure; the *white-white zone,* completely avascular and corresponding to the inner sharp edge of the meniscus, towards the intercondylar notch; and an intermediate area, called the *red-white zone*, which present midway characteristic of vascularization and cells phenotyping with the other two [Di Giancamillo et al. 2017].

Menisci are mainly composed of water (72% [Makris et al., 2011]), while their dry matter is composed of a collagen network, principally of type I and II, which entraps different phenotypes of cells in a rich-of-proteoglycans extracellular matrix (ECM). A recent study of our group, in pigs, demonstrated how collagen fibres are arranged in site-specific and depth-specific ways that allow meniscus to withstand tensile forces [Peretti et al., 2019], while proteoglycans (PGs), capturing and releasing water molecules via their negatively charged glycosaminoglycans (GAGs) terminuses, generate an osmotic pressure that allows menisci to withstand to compressive forces [Mahmood et al., 2018].

The different phenotypes of cells present throughout the tissue are distributed in a regional-specific manner and are responsible of the double nature of the meniscus. In the outer zone, fibrocyte-like cells (oval/fusiform in shape and with long cell extensions) reflect the fibrous pattern of meniscus, while, in the inner zone, chondrocyte-like cells (more rounded) are responsible of the cartilaginous-like behaviour of the tissue [as reviewed by Makris et al., 2011]. These regional variations are acquired during the latter stage of meniscal development, since initially cells show no phenotypical differentiation [Makris et al. 2011].

Collagen fibres are mainly of type I, however, some regional specializations characterize the distribution of the different types of collagens, with a predominance of collagen type I all over the red-red zone and the co-expression of both collagen type I and II (40:60) in the white-white zone [as reviewed by Makris et al., 2011]. Type I fibres are principally disposed following the circumferential shape of the meniscus in the outer zone [Skaggs et al., 1994], while collagen type II fibres are mainly expressed, as PGs, in the inner zone and in a radial direction [Skaggs et al., 1994; Rattner et al., 2011]. Collagen radial fibres envelope the circumferentially disposed collagen type I fibres to avoid their displacement when the application of a compressive force occurs [Rattner et al., 2011].

The peculiar arrangement of collagen bundles and the strict interconnection between collagen fibres (particularly those of type II) and GAGs lead to the elastic and viscoelastic properties that characterize the meniscus [as reviewed by McDermott et al., 2008].

During meniscal development, both cellularity and vascular supply continue to decrease, while, GAGs increase in the inner zone, and collagen fibres pass from a disorganized arrangement to the well-organized one previously described [as reviewed by Fox et al., 2012]. Furthermore, the collagen fibres present in the early stages of meniscal maturation are composed only of collagen type I, type II collagen will be expressed only in later stages of development and primarily in the inner zone [Melrose et al., 2005; Di Giancamillo et al. 2014].

Weight-bearing and joint motion during development are implied in determining the orientation of the collagen fibers [Fox et al. 2015] and the production of GAGs [Di Giancamillo et al. 2017].

Joint motion and the postnatal stress of weight-bearing are the principal factors that determine the phenotypical and architectural changes that characterize the maturation process of the meniscus. Mikic et al. (2000) described the high dependence of this structure on biomechanical force application for its correct development: these Authors observed the degeneration and the consequent disappearing of meniscus in only two days after joint immobilization in chicken embryos.

Furthermore, the impact of meniscus upon intra-articular wellness of knee joint is well explained by the demonstrating higher incidence of degenerative changes within the joint (such as osteoarthritis) consequent to its injury or removal [Fairbank, 1948; Vaquero and Forriol, 2016]. Meniscal tears are reported as spontaneous or, more frequently, secondary to ACL-deficiency lesions in both human and canine species. The choice of dog as one of the most utilized animal models in meniscal pathology and reparation is linked to the similarity in pathogenesis and anatomy between these two species [Krupkova et al., 2018].

Nowadays, different techniques are available to achieve the reparation (through sutures or implants) of the tissue or the complete replacement with tissue engineering (with allograft or scaffolds) of the meniscus [Vaquero and Ferriol, 2016]. However, these techniques are not avoided of technical critical points.

Tissue engineering targets to replace the damaged meniscus with a synthetic material that mimic its main functions [Makris et al., 2011]. This material may be an exact replica of the native tissue (with scaffolds and cells) or may just replicate its material properties (only with scaffolds). In the first case, the production of a native-like fibrocartilaginous ECM by the cells seeded in the scaffold is fundamental to achieve the complete substitution of the scaffold with neo-formed tissue [Makris et al., 2011].

One of the main problems associated to this approach is the achievement of the fibro-chondrocytic phenotype and its maintenance [Makris et al., 2011]. Thus, different stimuli, both biochemical (i.e. growth factors) and biomechanical, have been tried to enhance the production of meniscal ECM [Makris et al., 2011].

In this study the effect of compressive forces on neonatal meniscus will be evaluated to investigate the effect of this kind of stimulus on the whole tissue, focusing on meniscal cells maturation and collagen fibres arrangement.

2. Materials and methods

2.1. Description of the study

A litter of 12 Dobermann Pinchers, of approximately 2 months (57 days old) was recruited, each puppy presented a quadriceps contracture of the right hindlimb, while the left leg was clinically healthy. Owners decided for euthanasia at 57 days, so none of these animals died for causes related to the present study. Quadriceps contracture caused femoral growth disturbance (Fig. 1A, 1B) and distal epiphysis extra-rotation, that blocked the knee joint, with flexion impossibility. Nevertheless, these joints became a realistic model for compressive tissue engineering bioreactors. The presence of a healthy and a pathological limb represented the inclusion criteria for this study. There were utilized the right/affecting legs of this population like models of continue compression while the left/physiological legs were considered like controls.

The unit of Obstetric and Gynaecologic Clinic performed puppies post mortem evaluation, sampling and data registration. Puppies were refrigerated until 24 hours at 4°C and then stored at -20°C. The aetiology for the condition seems to be ascribable to an improper injection in the quadriceps muscle performed by the owner. However, all the animals were tested for the principal parasitic diseases (Neospora caninum and Toxoplasma gondii) associated to the quadriceps contracture and, once excluded these possible aetiologies, the puppies were submitted to a radiological evaluation to exclude a hind-limb fractures aetiology.

The congenital aetiology was excluded since the insurgence of the pathology started, simultaneously in all the puppies of the litter, the 10^{th} day after birth.

After all the clinical checks, a lateral arthrotomy were performed and the joints were dissected to isolate lateral and medial menisci of both legs (Fig. 1G). Thus, 5 animals were destined to morphological

evaluations (histology, histochemistry, immunohistochemistry) and the other 7 were destined to biomechanical and biochemical analyses.

In the text below, we will refer to a “compressed”, "pathological" or “affected” meniscus to describe menisci harvested from the right knees, while menisci harvested from the left joints will be labelled as “physiologic” or “healthy”.

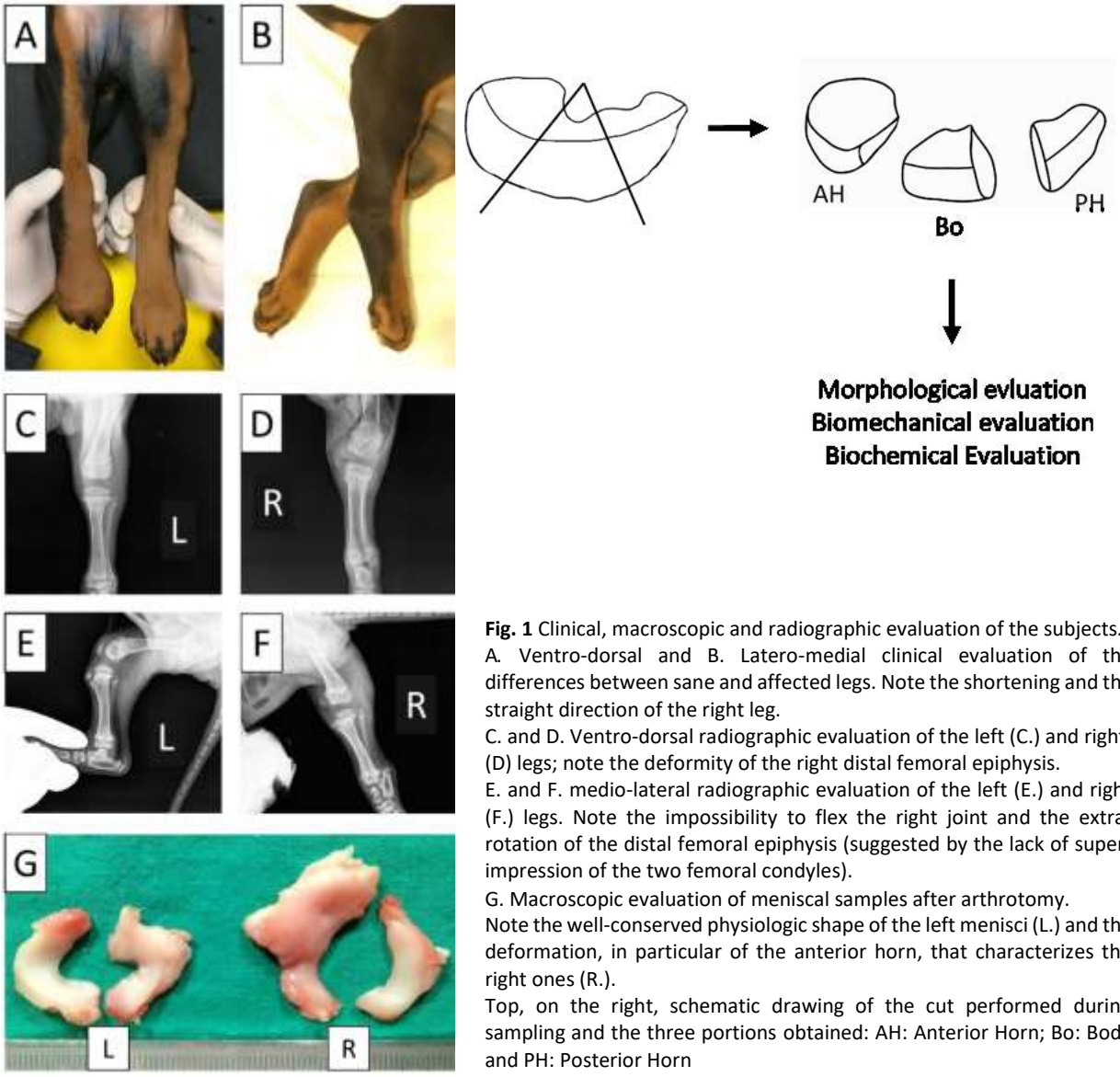

Fig. 1 Clinical, macroscopic and radiographic evaluation of the subjects.
A. Ventro-dorsal and B. Latero-medial clinical evaluation of the differences between sane and affected legs. Note the shortening and the straight direction of the right leg.
C. and D. Ventro-dorsal radiographic evaluation of the left (C.) and right (D) legs; note the deformity of the right distal femoral epiphysis.
E. and F. medio-lateral radiographic evaluation of the left (E.) and right (F.) legs. Note the impossibility to flex the right joint and the extra-rotation of the distal femoral epiphysis (suggested by the lack of super-impression of the two femoral condyles).
G. Macroscopic evaluation of meniscal samples after arthrotomy.
Note the well-conserved physiologic shape of the left menisci (L.) and the deformation, in particular of the anterior horn, that characterizes the right ones (R.).
Top, on the right, schematic drawing of the cut performed during sampling and the three portions obtained: AH: Anterior Horn; Bo: Body and PH: Posterior Horn

2.2. Morphological evaluation: histology, histochemistry and double immunohistochemistry

Both medial and lateral menisci harvested from the right and left knee of 5 animals (tot: 20 samples) were subdivided with two transversal cuts in three sections: the anterior and posterior horns and the central body (Fig. 1, top right). The obtained samples were immediately fixed in 10% buffered formalin. Then, dehydrated in a graded series of ethanol, cleared with xylene and embedded in paraffin. Serial longitudinal and transverse microtome sections (4 µm-thick) were obtained from each sample.

The differences in cellular maturation grade and matrix deposition between the two populations were evaluated trough histology (Haematoxylin-Eosin staining) and histochemistry (Goldner Masson's trichrome staining, Sirius Red staining and Safranin-O staining).

Haematoxylin-Eosin staining was performed to evaluate the eventual morphological differences between compressed and uncompressed meniscus cells and the arrangement of the collagen fibres. Sirius-red staining and Goldner Masson's trichrome staining were chosen to highlight the collagen fibres deposition and arrangements. Safranin-O staining was performed to highlight the GAGs presence within the tissue. Sirius-red histochemical-stained sections were analysed by means of polarized light microscopy to assess the spatial orientation of the fibres, highlighted by the birefringence of collagen fibres. The samples were analysed with an Olympus BX51 light microscope (Olympus, Opera Zerbo, Milan Italy) equipped with a digital camera.

Moreover, double immunofluorescence was performed to reveal the localization and the possible co-localization of the two main express types of collagens (type I and II). After rehydration, heat-induced antigen retrieval was performed as previously described [Di Giancamillo et al., 2017]. After washing three times in PBS (pH 7.4), sections were incubated with the first-step primary antiserum, 1:50 collagen I (Abcam, Cam- bridge, UK) for 24 hrs at 18–20°C, then washed in PBS, and subsequently treated with the Avidin–Biotin blocking kit solution (Vector Laboratories Inc., Burlingame, CA USA). The sections were then washed in PBS for 10 min. and incubated with a solution of goat biotinylated anti-rabbit IgG (Vector Laboratories Inc.), 10 lg/ml in Tris-buffered saline (TBS) for 1 hr at 18–20°C. After rinsing twice in PBS, the sections were treated with Fluorescein–Avidin D (Vector Laboratories Inc.), 10 µg/ml in $NaHCO_3$, 0.1 M, pH 8.5, 0.15 M NaCl for 1 hr at 18–20°C. For the second step of the double immunofluorescence procedure, sections were treated in a 2% hyaluronidase solution at room temperature for 30 min. The slides were subsequently treated with 1:50 anti-collagen II antiserum (Chondrex Inc., Redmond, WA USA). Sections were rinsed in TBS for 10 min. and incubated with 10 µg /ml goat biotinylated anti-mouse IgG (Vector Laboratories Inc.) for 1 hr at 18–20°C. The sections were then washed twice in PBS, and treated with Rhodamine–Avidin D (Vector Lab- oratories Inc.), 10 µg /ml in $NaHCO_3$, 0.1 M, pH 8.5, with 0.15 M NaCl for 1 hr at 18–20°C. Finally, slides with tissue sections were embedded in Vectashield Mounting Medium (Vector Laboratories Inc.) and observed using a Confocal Laser Scanning Microscope (FluoView FV300; Olym- pus). The immunofluororeactive structures were excited using Argon/ Helio–Neon–Green lasers with excitation and barrier filters set for fluorescein and rhodamine. Images containing superimposition of fluorescence were obtained by sequentially acquiring the image slice of each laser excitation or channel. In double immunofluorescence experiment, the absence of cross-reactivity with the secondary antibody was verified by omitting the primary antibody during the first incubation step.

2.3. Biomechanics

The elastic unconfined compressive modulus (Ec) was evaluated by means of EnduraTEC ELF® 3200 machine, equipped with a 22N load cell.

Medial and lateral menisci from both right and left knees of the remnant 7 puppies were transversally sectioned in the same three portions (anterior horn, body and posterior horn; tot: 84 samples), stored in saline solution NaCl 0.9% and frozen at -80°C until time of testing. At least 24 hours before test execution, samples were taken to a temperature of -24°C and then completely thawed at room temperature (23°C). Subsequently, for each zone, a cylindrical part perpendicular to the femoral and tibial surfaces was cut using a die cutter (Fig. 4, left upper side). The diameter of the specimens was dependent on the original meniscus. Before testing, dimensional measurements on the specimens were taken with a digital calliper. The samples were inserted into a Plexiglas cell and PBS solution was added into the cell to avoid dehydration of the specimen. The thickness of all samples was measured from the position of the testing machine actuator, after imposing a preload of approximately 0.01N. The sample was then subjected to a multi-ramp stress relaxation test, made of five increasing 4% strains at a velocity of 0.1%/s, followed by stress relaxation to equilibrium for 600 s. The compressive Young modulus, EC, was obtained for each ramp from the equilibrium data as the ratio between values of relaxation stress and the corresponding values of strain.

2.4. Biochemical analysis

Biochemical analysis was immediately performed on the same samples previously analysed by biomechanical tests. Meniscal portions were digested in papain (Sigma) for 16–24 h at 60°C; the digestion solution was composed of 125 mg/mL of papain (Sigma) in 100mM sodium phosphate, 10mM sodium EDTA (Sigma), 10mM cysteine hydrochloride (Sigma), 5mM EDTA adjusted to pH 6.5 and brought to 100mL of solution with
distilled water. After the digestion, the samples were stored at – 80°C until analysis. Aliquots of the digested samples were assayed separately for proteoglycan and DNA contents.

Proteoglycan content was estimated by quantifying the amount of sulphated glycosaminoglycans using the 1,9-dimethylmethylene blue dye binding assay (Polysciences, Inc.) and a microplate reader (wavelength: 540 nm). The standard curve for the analysis was generated by using bovine trachea chondroitin sulphate A (Sigma). DNA content was evaluated with the Quant-iT Picogreen dsDNA Assay Kit (Molecular Probes, Invitrogen) and a fluorescence microplate reader and standard fluorescein wavelengths (excitation
485 nm, emission 538 nm, cut-off 530 nm). The standard curve for the analysis was generated using the bacteriophage lambda DNA supplied with the kit.

2.5 Statistical Analysis

Statistical analyses of the biomechanical and biochemical data were analysed with 2-ways ANOVA with side (left and right) and meniscal portions (anterior horn, body, posterior horn) as main factors. The statistical analysis was performed using the general linear model of the SAS (version 8.1, Cary Inc., NC). The meniscus was considered to be the experimental unit of all response variables. The data were presented as least squared means ± SEM. Differences between means were considered significant at $P < 0.05$ and highly significant at $P < 0.01$.

3. Results

3.1. Consideration about the pathological condition

Quadriceps contracture syndrome is a rare pathology that may have different aetiologies, including parasitic disease, congenital affection and femoral fracture. To exclude femoral fracture as aetiology, standard ventro-dorsal (Fig. 1C, 1D) and latero-medial (Fig. 1E, 1F) projections were performed, with negative results.

This syndrome is characterized by the fix hyperextension of both knee and ankle joints (as these joints are characterized by a consensual mechanisms of flexion/extension), consequently, the affected knee is unable to flex (as can be note confronting the two medio-lateral radiographies in which is possible to note the inability to flex, in the consuetudinary 90° angle, the right knee (Fig 1E *vs* 1F)).

Usually, this pathology affects growing animals and in these cases deformation and shortening of the affected limb occur (Fig. 1A-F). The deformation of the limb is consequence of the increase of the bone segments length associated to the articular block and lead to an extra-rotation of the femoral distal epiphysis.

In this way a constant compression, that derives both from the longitudinal growth of the bone segments and from the weight bearing, is applied upon the structure that resides within the knee. On the other hand, tensile forces generated by the flexion/extension of the joint are practically zeroed.

Due to the particular conformation of the deformed femur, the most compressed areas in the affected limbs result to be the external area of the menisci, contrarily to what is observed in the normal ones, in which the most compressed area is the inner zone.

This condition lead to macroscopic differences in meniscus shape, with the physiologic menisci that show the consuetudinary semilunar and wedge-like shape and the affected ones that show a deformed shape (Fig. 1G, right side).

3.2. Morphological evaluation: histology, histochemistry and double immunohistochemistry

Collagen fibres arrangement and cellular shape were evaluated by means of Haematoxylin-Eosin staining (Fig. 2A, 2E), Sirius-red staining (Fig. 2C,2G) and Goldner Masson's trichrome staining (Fig.2B, 2F).

Other sections were analysed by means of polarized light microscopy after Sirius-red histochemical staining to assess the spatial orientation of the fibres, highlighted by the birefringence of collagen fibres (Fig. 2D, 2H).

Typically, collagen fibres are characterized by a wavy aspect as they are composed of crimps. These crimps are well evident in the histologic (Fig. 2A), in all the histochemical staining (Fig. 2B, 2C) and in the polarized microscopy evaluation (Fig. 2D) of the physiologic meniscus. Moreover, it is possible to note how the collagen bundles follow a well-ordinated and unidirectional arrangement of in the physiologic meniscus (Fig. 2A-D). The collagen fibres are well highlighted by the polarized light and show the characteristic anisotropic behaviour that allows to note the fibres' crimps (Fig. 2D). On the other hand, the compressed meniscus is characterized by a chaotic disposition of the collagen fibres which look stretched without their typical wavy appearance (Fig. 2E-H) and show the loss of their distinctive anisotropic behaviour (Fig. 2H).

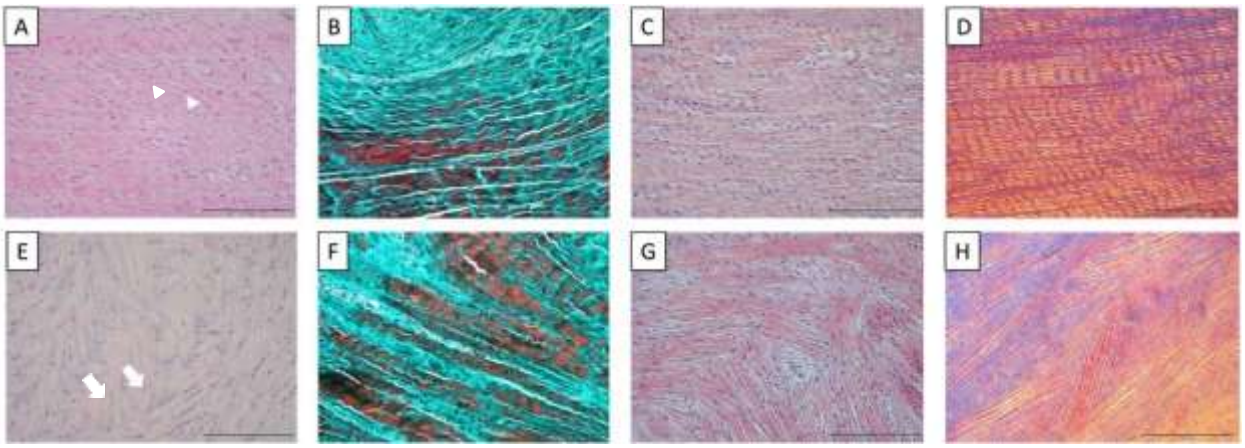

Fig. 2. Histologic (A; E) and histochemical (B; C; D; F; G; H) staining of the left anterior horns (AH) of healthy and pathological menisci; longitudinal section. A; B; C; D. Haematoxylin-Eosin (A); Goldner-Massons' Tricromic (B); Picrosirius red (C) and Polarized light microscopy (D) of the healthy meniscus; E; F; G; H. Haematoxylin-Eosin (E); Goldner-Massons' Tricromic (F); Picrosirius red (G) and Polarized light microscopy (H) of the affected meniscus. Note the different arrangements of the collagen fibres between healthy and affected menisci and the different shape of cells: fusiform (white arrow-head) in the healthy meniscus and more rounded (white arrow) in the compressed one.

Cells show a fusiform shape in the healthy meniscus (Fig. 2A, white arrow-head) and a more rounded shape in the compressed meniscus (Fig. 2E, white arrow).

Safranin-O staining was performed to highlight the GAGs presence within the tissue (orange/pink) and to evaluate the morphology of the cells in the three meniscal zones.

The physiological meniscus shows a higher concentration of GAGs in the inner zone (Fig.3A, asterisk), while the outer zones present only a sporadic staining for GAGs (Fig. 3B and 3C). Even in these slides, the cells present in the inner zone show a more rounded shape when confronted with the cells present in the other two zones which are more fusiform (Fig. 3A, arrow *vs* 3B and 3C, arrow-heads). The compressed meniscus shows a higher concentration of GAGs in the two outer zones while the inner zone shows only a pale staining (Fig. 3B and 3C *vs* 3A). Differently to what previously observed in the left knee meniscus,

the populations that reside in the different area present a more rounded shape in all the three areas (Fig. 3D, 3E, 3F, arrows), with a higher incidence of fusiform cells only in the outermost zone (Fig. 3F, arrow-head).

Immunohistochemistry was executed to evaluate the different expression of the two principal types of collagens (I and II) present in the menisci as well as the cellular morphology (Fig. 4A-F).

Collagen type I (Fig. 4A, marked in red) and II (Fig. 4B, marked in green) are co-expressed in the matrix and present a poorer nuclear expression in physiological meniscus (Fig. 4C). Nuclear shape (Fig. 4A-C) is elongated and recalls the aspect previously described with histologic and histochemical staining. The counterpart compressed meniscus is characterized by a predominance of collagen II (Fig. 4F) and a clear nuclear localization (Fig. 4E, 4F). Collagen I showed a very poor expression both in the matrix and nuclei (Fig. 4D). The nuclei present in the compressed menisci showed a more rounded shape (Fig. 4D-F) respect to the elongated nuclei present in the physiological ones (Fig. 4A-C).

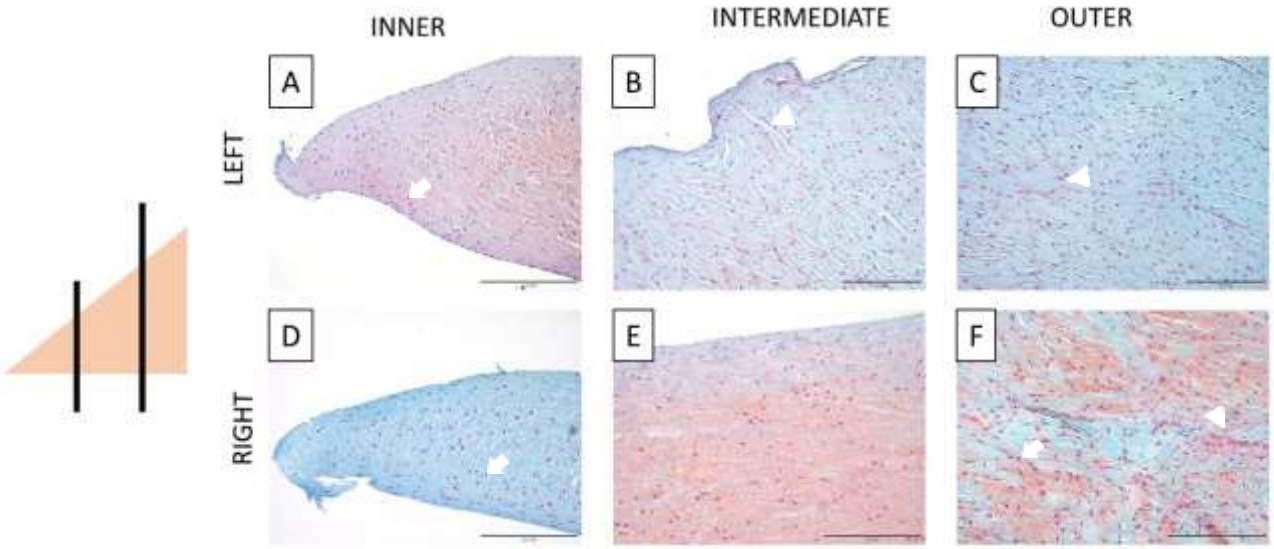

Fig. 3 Histochemical Safranin-O staining. On the left, a schematic draw that explain the type of section (transversal) and the three areas of the meniscus (inner, intermediate and outer). A; B; C. staining of the inner (A), intermediate (B) and outer (C) zones of the left meniscus. D; E; F. staining of the inner (D), intermediate (E) and outer (F) zones of the affected meniscus. Note the differences between the localization of the GAGs (stained in orange, asterisk) in the matrix of the two menisci (in the inner zone for the sane meniscus and in the intermediate and the outer zones for the affected one) and the different shapes of cells' nuclei (fusiform in the outer zones of the healthy meniscus and in the outermost area of the compressed one, arrow-heads, and more rounded in the inner area of the healthy and compressed meniscus and in the more compressed intermediate and outer zone of the affected meniscus, arrow.)

3.3. Biomechanics

Unconfined Young's Ec in response to compression was tested in order to assess eventual differences between lateral and medial menisci (Fig. 5B) and between the anterior and posterior horns (Fig. 5C), always considering the difference between compressed and physiologic menisci (Fig. 5A).

Pooled healthy menisci showed a higher Ec respect to the affected ones ($p<0,05$). Medial and lateral physiologic menisci show higher Ec confronting with the counterparts of the pathological ones ($p<0,05$ for both the comparisons).

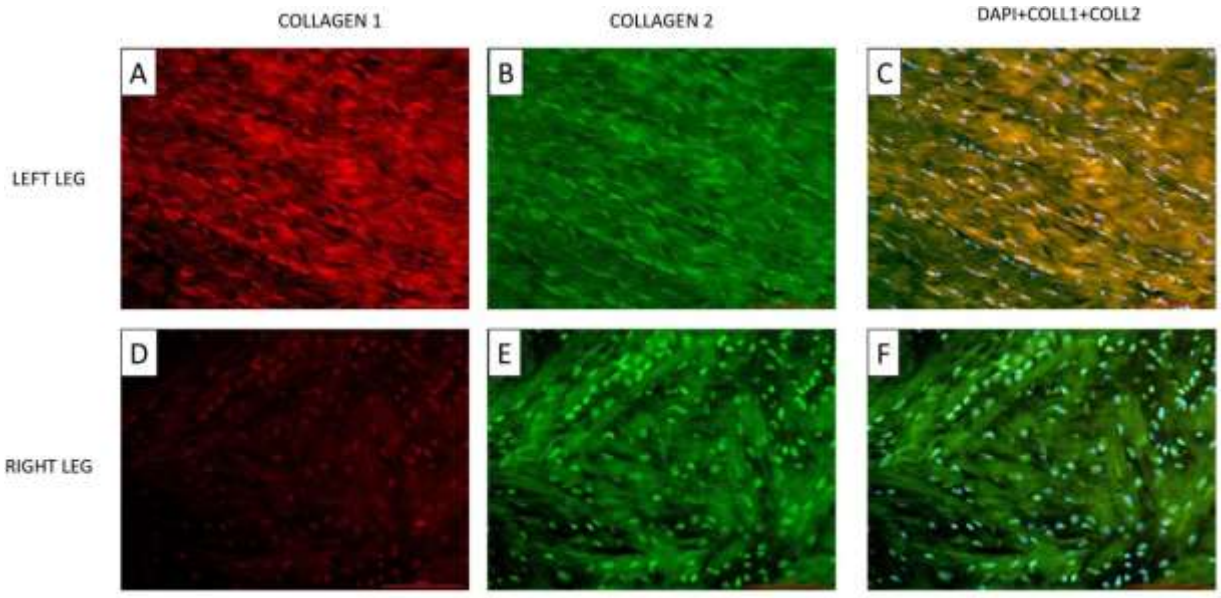

Fig. 4. Double immunohistochemistry of the anterior horns of the healthy (left) and compressed (right) menisci; longitudinal section. A and D. Collagen type I expression in healthy (A) and affected (D) menisci. B and E. Collagen type II expression in healthy (B) and affected (E) Menisci. C and F. Co-expression of collagen type I and II in healthy (C) and affected (F) menisci. Note the round shape nuclei and the random arrangement of the collagen fibres present in the affected menisci (D; E; F) vs the elongated nuclei and the well-organized arrangement present in the healthy ones (A;B;C). Red: collagen type I; Green: collagen type II; Yellow: co-expression of collagen type I and II; light blue: DAPI.

However, only the difference between the horns of the lateral menisci are statistically significant with the healthy menisci portions that show the higher moduli values (for both, anterior and posterior horns, $p<0,01$), while, in the medial compartment, no statistically significative results are shown considering the two horns. Nevertheless, in all the comparisons the physiological meniscus shows a higher Elastic modulus.

3.4. Biochemical analysis

The presence of GAGs (Fig. 6A) and the quantification of cells (DNA; Fig. 6B) within the meniscus was analysed by means of biochemical essays, always considering the two sides. Furthermore, the GAGs/DNA ratio (Fig. 6C) was also calculated to highlight the grade of maturation of the cells in relation to their capacity to produce GAGs.

GAGs quantification revealed a higher concentration of this proteins in the left menisci respect to the right one, even if with no statistically significant differences (Fig. 6A). A decrease in cells number is also seen, with a significant statistical difference ($p<0,05$), in the compressed menisci (Fig. 6B). The GAGs/DNA ratio showed no statistical difference, but a higher value in the menisci harvested from the right knee (Fig. 6C).

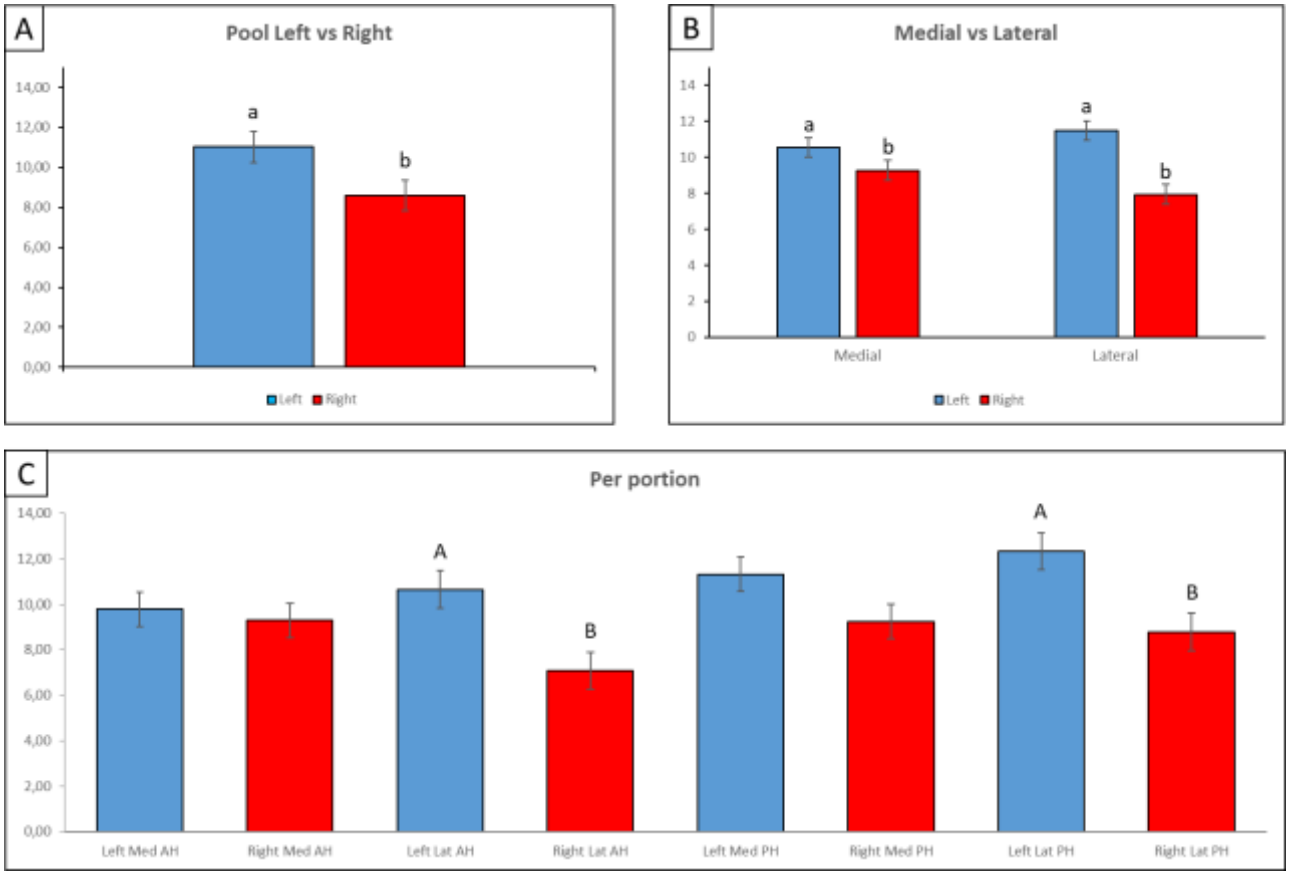

Fig. 5 Biomechanical evaluation of the Young's Elastic Modulus in compression (EC). Blue: Left/healty menisci. Red: right/affected menisci. a, b: p<0,05; A, B: p<0,01.

When the different portions of each side meniscus are considered two trends are principally showed: in the physiologic meniscus, no difference among anterior horn, body and posterior horn is found (Fig. 6D, left side),

while in the pathologic meniscus a dichotomic pattern is present, in which the anterior horn showed a higher GAGs concentration respect to the other two portions (Fig. 6D, right side; p<0,01).This dichotomic pattern is conserved even in the cellularity results of the compressed meniscus (Fig. 6E, right side), contraposed to the results of the healthy menisci characterized by a smother differentiation among the three areas, whit a higher numerosity in the anterior horn respect to the posterior horn and an intermediate value expressed in the body section (Fig. 6E, left side).

Intriguingly, the GAGs/DNA ratio showed a higher value in the anterior horn of the compressed meniscus (respect to the other two portions) while its left counterpart showed the smaller value (Fig. 6F), however, the differences described represent only trends, since none of these showed statistical significance.

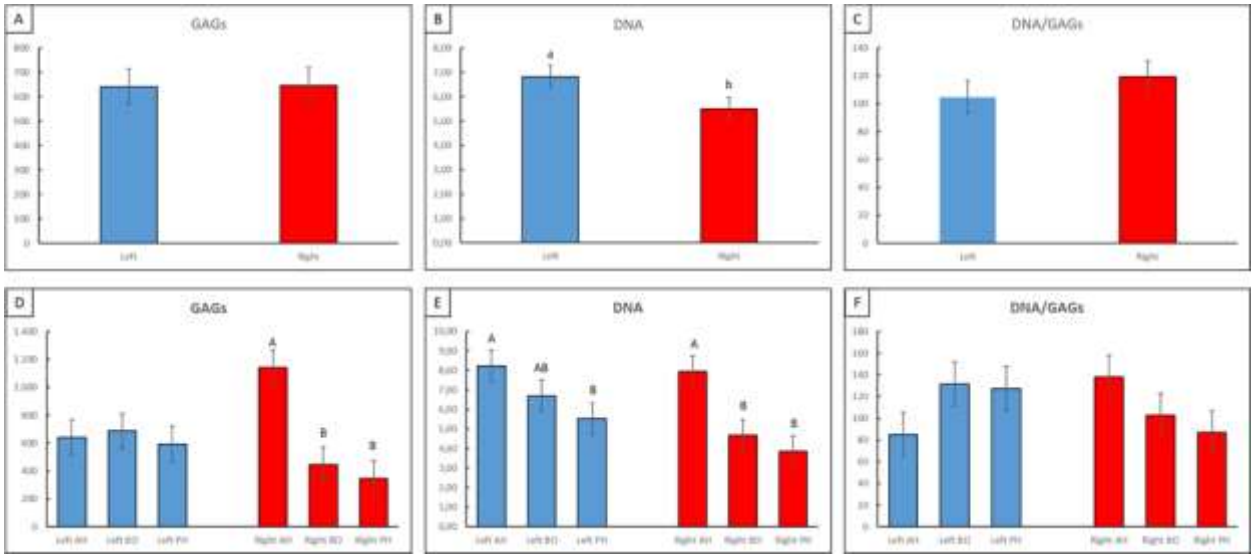

Fig. 6 Biochemical essay: GAGs (A, D), DNA (B, E) and GAGs/DNA ratio (C, F) analysis for the whole left and right menisci (A, B, C) and for the different portions (D, E, F). Blue: left/healthy meniscus; red: right/affected meniscus. a, b: p<0,05; A, B:

4. Discussion

Mechanical stresses are fundamental for the health and function of the meniscus [Krupkova, et al., 2018], in both humans and animal models [Dowdy PA et al., 1995].

It has been demonstrated how biomechanical stimuli are essential factors in the maturation of meniscal cells phenotype and consequently in the production of meniscal ECM in sheep and pigs [Meller et al., 2009; Di Giancamillo et al. 2014].

Lack of physiologic movements lead to disuse atrophy, characterized by the loss of proteoglycans, in mature meniscus (as reviewed by McNulty et al., 2015), and to the degeneration and the disappearing of this structure during embryologic development as observed in chickens [Mikic et al., 2000].

Meniscal cells may react to mechanical stimulation either with an anti-inflammatory or pro-inflammatory response. Moreover, biomechanical stimulation influences the balance of ECM turnover toward anabolism or catabolism. These opposite behaviours depend on magnitude, frequency, duration and type (static or dynamic) of the force applied [McNulty et al. 2015; Makris et al. 2011].

It has been demonstrated as dynamic compression at 10% strain may stimulate either anabolic and catabolic metabolism; with the suppression of pro-inflammatory mediator [as reviewed by Aufderheide and Athanasiou, 2006, McNulty et al. 2015 and Gupta et al., 2008].

Instead, clear pathologic effects, that lead to proteoglycans breakdown, have been reported after the application of higher strain (20%) with upregulation of matrix metalloproteinase (MMP)-1, MMP-3, MMP-13, ADAMTS-4, inducible nitric oxide synthase (NOS2), interleukin-1α (IL-1α) mRNA, and Nitric Oxide (NO) [as reviewed by McNulty and Guilak, 2015].

Pathological models of abnormal joint loading resulted in catabolic and pro-inflammatory responses with a significant decrease in cell viability and a trend towards increased pro-inflammatory mediators (as NO) [as seen in leporine model by Killian et al., 2014].

Nevertheless, studies on overloading models reported no macroscopic damage to the tissue, no detrimental effect on the sulphated-glycosaminoglycan (sGAG) content and release, the downregulation of many catabolic and pro-inflammatory genes, and no effect on the mechanical properties of the tissue. However, overloading caused large regions of cell death at the surface of the tissue [Kisiday et al. 2010]. Finally, Ballyns and Bonassar (2011) reported that a dynamic unconfined compression load (15% strain and 1 Hz 3 times/week for up to 6 weeks) lead to an enhancement of the extracellular matrix content and compressive modulus after 2 weeks and a more mature matrix (characterized by increase in collagen bundle formation, GAGs content, collagen content, and compressive modulus) was obtained by Puetzer and colleagues (2012) after 2 weeks of loading followed by 4 weeks of static culture [Puetzer et al., 2012].

Regarding the present study, the first consideration that must be done is that an accidentally occurred pathologic condition in an animal model was considered as a substitute of a bioreactor that applies a dynamic compressive force (the alternation of load- and no-load-bearing) in association to a static compression (due to the continuous growth of the bone segments [Henderson and Carter, 2002], that compressed the meniscus between the femur condyles and the tibial plateau), thus, menisci in this study were always subjected to a compressive forces, with no relax periods. Unfortunately, even if this situation agrees with the 3R principles (Reduce, Reuse and Refinement), it is limited by the impossibility to determine the precise mechanical environment of the menisci and may be further complicated by the effect of systemic factors.

Consequently, even if we can suppose a clear effect of the compression on our samples, we cannot quantify the entity of this compression and the frequency of the dynamic compression due to the intermittent periods of weight-bearing and non-loading.

However, *in vivo* animal studies represent the most physiologically relevant model systems and allow the study of long-term (i.e., weeks to years) effects associated with development, remodelling, or repair. Furthermore, we utilized tissue explants, which provide a reservoir of natural committed cells that preserved their natural relationship with and within the extracellular matrix, thus the effect of the compressive force can be evaluated without any bias that may related to a synthetic composition or to the lack of the ECM.

Knowing all this, the results of the present study may still add new tesserae to the evaluation of the effect of biomechanical stimulation upon meniscal tissue and cells.

The morphological evaluation performed by histochemical staining confirmed that GAGs are produced in the most compressed areas: physiologically, the inner area of the meniscus is considered as the most compressed, and richer of GAGs [Melrose et al. 2005] (as confirmed by our Safranin-O staining of the physiological meniscus, Fig. 3A); however, due to the extra-rotation and the deformation of the distal femoral epiphysis consequent to the quadriceps contracture (Fig.1D, 1F), a lateral shifting of the point of

higher compression to the meniscal outer zones can be suggested and seems to be confirmed by our results (Fig. 3E, 3F).

Moreover, our morphological evaluation (both histology and histochemistry) shows that cells react to the compressive stimulus triggering their maturation: in the over-compressed area of the meniscus, cell's nuclei acquired a rounded shape (Fig. 2 and Fig. 3) that is typical of meniscal fibro-chondrocytes, a phenotype that physiologically resides in the inner and cartilaginous-like portion of the meniscus; furthermore, they produce a larger quantity of GAGs, respect to the uncompressed areas of the same samples (Fig. 3D, inner area of the compressed meniscus) or to the physiologically loaded counterparts (Fig. 3B, 3C outer area of the uncompressed).

The apparently contrasting round shaped cells present in the inner zone (Fig 3E, arrow) and the fusiform shaped cells present in the outermost area (Fig. 3F, arrow-heads) of the compressed meniscus, may be explained by an initial physiological regionalization (as described by Makris et al., 2011 and Fox et al., 2015) that may have been interrupted since the pathology occurred, but not completely lost.

On the other hand, differences are also present considering the expression and the arrangement of collagen fibres. Our histological and histochemical results show how, in the physiologic menisci, the collagen fibres display an ordered arrangement which follows the hypothetical tensile force generated by normal locomotion (Fig. 2A-D), while, in the pathological menisci, the collagen fibres possess a chaotic and not functional arrangement (Fig. 2E-H). Moreover, the typical wavy aspect of collagen fibres, characterized by the presence of crimps and responsible of the elastic properties of meniscus, is displayed only in the physiologically loaded menisci (Fig. 2D), while the over-compressed ones are composed of stretched fibres, that have lost their crimps and their elastic ability (Fig. 2H).

However, analysing the collagen fibres, we found that those deriving from the healthy menisci are almost entirely composed by collagen type I (Fig.4A) as it is described in physiologic menisci of young sheep and pigs [respectively, Melrose et al. 2005 (sheep); Di Giancamillo et al. 2014 (pigs)].

Biomechanical and biochemical essays were performed trying to quantify these apparent differences and to characterize their functional role in meniscal tissue.

Biomechanical evaluation of the meniscal elastic modulus draws a picture characterized by the inability of the pathologic menisci to withstand to the compressive force, respect to the physiologic ones: Ec modulus of compressed menisci are lower than those of the healthy ones, when side (left vs right; Fig. 5A), compartments (medial or lateral; Fig. 5B) and portions (anterior or posterior horns; Fig. 5C) are considered.

Furthermore, biochemical quantification of meniscal tissue depicts a complex scene in which no differences are seen in the quantity of GAGs produced in the whole tissue (Fig. 6A) respect to the sides, but it is possible to observe a decrease in cell density (Fig. 6B; $p<0,05$) and a higher GAGs/DNA ratio (Fig. 6C; no significant statistical difference) which may be read as a switch towards a mature tissue

(characterized by small amount of cells that produce a rich ECM (Di Giancamillo et al. 2017). This pattern is mainly expressed in the anterior horn (with a dichotomic differentiation between anterior horn and the other two portions; Fig. 6D-E; $p<0,01$, and a higher value for the ratio, Fig. 6F) that probably corresponds, in the pathologic menisci, to the most compressed area.

Taken together the result of the biomechanical and biochemical analyses suggest that even if the over-compressed meniscus shows a precocious maturation of the resident cells (fibro-chondrocytic-like appearance) and consequently of the matrix (i.e. collagen type II expression, higher GAGs quantity), the lack of a well-organized collagen network lead to the development of an incompetent tissue. Different Authors, described the importance of the collagen network in response to the application of tensile forces through the generation of the so-called hoop stress [Krupkova et al., 2018]; Other Authors described the deep association of collagen type II fibres and GAGs [Valyaveettil et al., 2005]; however, to our knowledge, this is the first study that demonstrated the role of this collagen/GAGs relationship in the achievement of the typical anisotropic and elastic behaviour of meniscal tissue in response to a compressive stimulus.

An application of a compressive force for a short period could be useful in meniscal tissue bio-engineering to trigger a quicker achievement of the mature fibro-chondrocytic phenotype from committed meniscal cells, and consequently the earlier production of the fundamental ECM, in order to improve cellular viability and adhesion of these cells within a potential synthetic scaffold.

4. Conclusion

This investigation shows how native meniscal tissue varies under different biomechanical stimuli, producing useful background information for further tissue engineering applications.

The results of this study suggest that a continue compressive force, applied to the native meniscal cells, triggers an early maturation of the cellular phenotype, at the expense of the proper organization of collagen fibres.

Nevertheless, an application of a compressive force could be useful in meniscal tissue bio-engineering acting as a booster in the achievement of the mature cellular phenotype, and consequently the earlier production of the fundamental ECM, in order to improve cellular viability and adhesion of the cells within a synthetic scaffold.

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Conflict of interests:

Authors declare no conflict of interests.

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4.3 HYPOXIA AS A STIMULUS UPON NEONATAL SWINE MENISCUS CELLS: HIGHWAY TO PHENOTYPIC MATURATION OF MENISCAL FIBRO-CHONDROCYTES?

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Abstract:

Great interest, in both human and veterinary medicines, is reserved to the treatment of the injuries of the meniscus: however, until now, there are no perfect solutions for the regeneration, or the replacement, of this tissue once injured. This work is focused on the utilization of an environmental factor like hypoxia in meniscal tissue culture, in order to evaluate if it could be utilized to improve meniscal culture with a view to tissue engineering. Ninety menisci from neonatal pigs (day 0) were harvested and cultured under two different atmospheric conditions (hypoxia with 1% O_2 and normoxia) until 14 days. Samples were analysed at 0, 7 and 14 days through histochemical (Safranin-O staining), immunofluorescence and RT-PCR (Sox-9, Hif-1a, Collagen I and II, both methods) and biochemical (DNA, GAGs, DNA/GAGs ratio) techniques to record any possible differences in maturation of meniscal cells. Safranin-O staining allowed to show an increment in matrix deposition and round-shape "fibro-chondrocytic" cells quantity of hypoxia-cultured menisci respect to controls under normal atmospheric conditions. The same maturation shifting was observed by means of immunofluorescence and RT-PCR analysis, characterized by an increment of Sox-9 and collagen II, moving from day zero to 14-days under hypoxic environment, and by biochemical analysis, with an increment of DNA/GAGs ratio typical of mature meniscal tissue (characterized by few cells and much GAGs). This study shows that hypoxia can be considered as a booster to achieve meniscal cells maturation and opens considerably opportunities in the field of meniscus tissue engineering.

Keywords: Glycosaminoglycans, GAGs, pig cells, hypoxia, meniscus, collagen type II, SOX-9

1. Introduction:

Regenerative medicine is trying to achieve the goal of replacement or regeneration of the meniscus since its fundamental functions in the knee joint are been revealed [Fairbank, 1948]. Even if a great amount of efforts were made in this field, the different techniques adopted trying to replace or regenerate meniscus, still present some criticisms and pitfalls that bring to the not-achieving of the final goal [Vaquero and Forriol, 2016; Makris et al., 2011].

Main criticisms are the easy tendency to dedifferentiation of cells during their culture (with a decrease of collagen II and aggrecan), the poorer biomechanical properties of meniscal substitutes and the missed integration of cultured cells on scaffolds [Vaquero and Forriol, 2016 and Makris et al., 2011]. These could be due to the lack of proper technique or culture conditions of meniscal tissue or cells.

Only in the last years, techniques that consider the physiological environment of joint space in which meniscus is located were considered. For example, articular space is characterized by hypoxia (1-9 % O_2) [Lund-Olesen, 1970; D'Ippolito et al., 2006; Khan et al., 2007] that rarely is applied to usual normoxic (˜21% O_2) meniscus and articular cartilage tissue or cells cultures. Thus, menisci (in particular the inner / avascular zones) and cartilage are usually cultured in a hyperoxic environment instead of the physioxic environment that may be replicate by hypoxic culture, and this may somewhat false the obtained results and may explain some discrepancies which has been observed in the passage from *in vitro* to *in vivo*.

Cartilaginous cells can survive to the otherwise inhospitable environment thank to hypoxia-inducible factor-1 alpha (HIF-1α) an essential factor for chondrocyte survival and cartilage homeostasis [Pattapa et al. 2019].

Hypoxia-inducible factors (HIFs) are oxygen-sensitive transcriptional complexes constituted by α- and ß-subunits that activate diverse pathways regulating cellular proliferation and metabolism [as reviewed by Vettori et al. 2017 and McGarry et al., 2018]. Under high concentration of oxygen conditions (i.e. normoxia), the HIF-1α transcriptional subunit is recognized by prolyl-hydroxylases and degraded via the Von Hippel Lindau (VHL)-mediated ubiquitin proteasome pathway; however, under lower oxygen concentration conditions (i.e. hypoxia), HIF-1α is stabilized and translocates to the nucleus to exert its transcriptional activity [Vettori et al. 2017].

Most studies of mesenchymal stem cells chondrogenesis, either in pellet or scaffold cultures, indicate an increase in chondrogenic genes (SOX9, COL2A1 and ACAN [as reviewed by Pappata et al., 2019]) and matrix formation under physioxia [as reviewed by Pappatta et al., 2019].

In particular, HIF-1 interacts with SOX9 to upregulate collagen type II (COL2A1) and aggrecan (ACAN) genes (both characteristic of the cartilaginous phenotype), whereas it has been demonstrated a downregulating of collagen type X (COL10A1; a marker of endochondral ossification) expression under physioxia [as reviewed by Pattapa et al., 2019].

Hypoxic culture (from 1% to 13% O_2) is associated to similar results even in meniscus with an enhancement of fibro-chondrocytic ph3enotypical traits in human meniscal cell aggregates [Adesida et al., 2007]. In

human meniscus cell aggregates, physioxia induces an increment of SOX-9, linked to the post-transcriptional effect of HIF-1α, and consequently of collagen type II, both characteristics of cartilaginous tissue and signs of maturation of fibro-chondrocytes in meniscus [Adesida et al., 2007].

The application of hypoxia may be a fundamental step in the tissue culture of meniscus avoiding the dedifferentiation and promoting the subsistence of phenotypic features in meniscal samples, as suggested in previous works on cartilage and meniscal cells cultures [as reviewed by Pattapa et al., 2019; Adesida et al., 2006, 2007 and 2012]. Nevertheless, the effect of this environmental condition upon the whole meniscal tissue, its cellular phenotypes and extracellular matrix composition, is still matter of interest and the application on an immature tissue, composed of committed, but not already functional, cells, is not already evaluated.

In this study, we have assessed the pure effect of hypoxia (1% O_2) upon differentiation, proliferation and endogenous activation of committed cells within the whole neonatal meniscal tissue, characterized by the native relationship between cells and extracellular matrix.

2. Materials and methods

2.1. Study design:

Neonatal menisci (n: 90) were collected from stillbirth swine provided by a local breeding farm. The Ethic Committee of the University of Milan (OPBA, 58/2016) approved the use of cadavers for research purpose; furthermore, all the animals are dead for causes not related with the present study.

Eighteen menisci were analysed as common starting point and the remaining 72 were split into two groups each of 36 menisci: hypoxia, i.e. with a tension of oxygen of 1%, and normoxia, i.e. normal (atmospheric) oxygen conditions (˜21% O_2), as described in Fig. 1.

Menisci were stored within 6-well plates in a static culture with Dulbecco's modified Eagles medium (DMEM) supplemented with 20% Fetal Bovine Serum (FBS), 1,000 unit/mL of penicillin/streptomycin and 25 mg/mL fungizone [Dai et al. 2013; Narita et al. 2009] at 37°C, changed every three days.

Hypoxia group's menisci were stored in a controlled atmosphere chamber with low tension of oxygen (1% O2), at 37°C. Medium substitutions (each 3 days) and midway and final sampling (at 7 and 14 days) were performed under the same hypoxic conditions.

Normoxic group's menisci were stored under standard condition in uncontrolled atmosphere condition (with approximately 21% of O_2) in a sterile hood, at 37°C.

Eighteen menisci were withdrawn from each group after 7 days and 14 days of differentiated clotures. Samples were analysed by morphological analysis (histochemistry and immunofluorescence), biochemical analysis and real time PCR techniques (n: 6, per each group and techniques). No differences regarding medial and lateral menisci were considered.

2.2 Morphological evaluation (Histochemistry and immunofluorescence)

Samples were fixed in buffered 10% formalin (Bio-Optica, Milan, Italy) for 24 h, dehydrated and embedded in paraffin (total number of specimens= 30; 6 per each treatment and time points). Microtome longitudinal sections (4-µm thickness) of the whole menisci were analysed with Safranin-O staining (SO) for describing both the presence of GAGs in the matrix and the meniscal structure [Peretti et al., 2019]. Immunofluorescence was utilized with the aim of revealing the possible co-localization of collagen type II and SOX-9. After rehydration, heat-induced antigen retrieval was performed as previously described [Deponti et al., 2012; Di Giancamillo et al., 2017]. After washing three times in PBS (pH 7.4), sections were incubated with the first-step primary antiserum, 1:100 SOX9 (Abcam, Cambridge, UK) for 24 hrs at 18–20°C, then washed in PBS, and subsequently treated with the Avidin–Biotin blocking kit solution (Vector Laboratories Inc., Burlingame, CA USA). The sections were then washed in PBS for 10 min. and incubated with a solution of goat biotinylated anti-rabbit IgG (Vector Laboratories Inc.), 10 µg/ml in Tris-buffered saline (TBS) for 1 hr at 18–20°C. After rinsing twice in PBS, the sections were treated with Fluorescein–Avidin D (Vector Laboratories Inc.), 10 µg/ml in $NaHCO_3$, 0.1 M, pH 8.5, 0.15 M NaCl for 1 hr at 18–20°C. For the second step of the double immunofluorescence procedure, sections were treated in a 2% hyaluronidase solution at room temperature for 30 min. The slides were subsequently treated with 1:50 anti-collagen II antiserum (Chondrex Inc., Redmond, WA USA). Sections were rinsed in TBS for 10 min. and incubated with 10 ug/ml goat biotinylated anti-mouse IgG (Vector Laboratories Inc.) for 1 hr at 18–20°C. The sections were then washed twice in PBS, and treated with Rhodamine–Avidin D (Vector Laboratories Inc.), 10 µg/ml in $NaHCO_3$, 0.1 M, pH 8.5, with 0.15 M NaCl for 1 hr at 18–20°C. Finally, slides with tissue sections were embedded in Vectashield Mounting Medium (Vector Laboratories Inc.) and observed using a Confocal Laser Scanning Microscope (FluoView FV300; Olympus). The immunofluororeactive structures were excited using Argon/Helio–Neon–Green lasers with excitation and barrier filters set for fluorescein and rhodamine. Images containing superimposition of fluorescence were obtained by sequentially acquiring the image slice of each laser excitation or channel. In double immunofluorescence experiment, the absence of cross-reactivity with the secondary antibody was verified by omitting the primary antibody during the first incubation step.

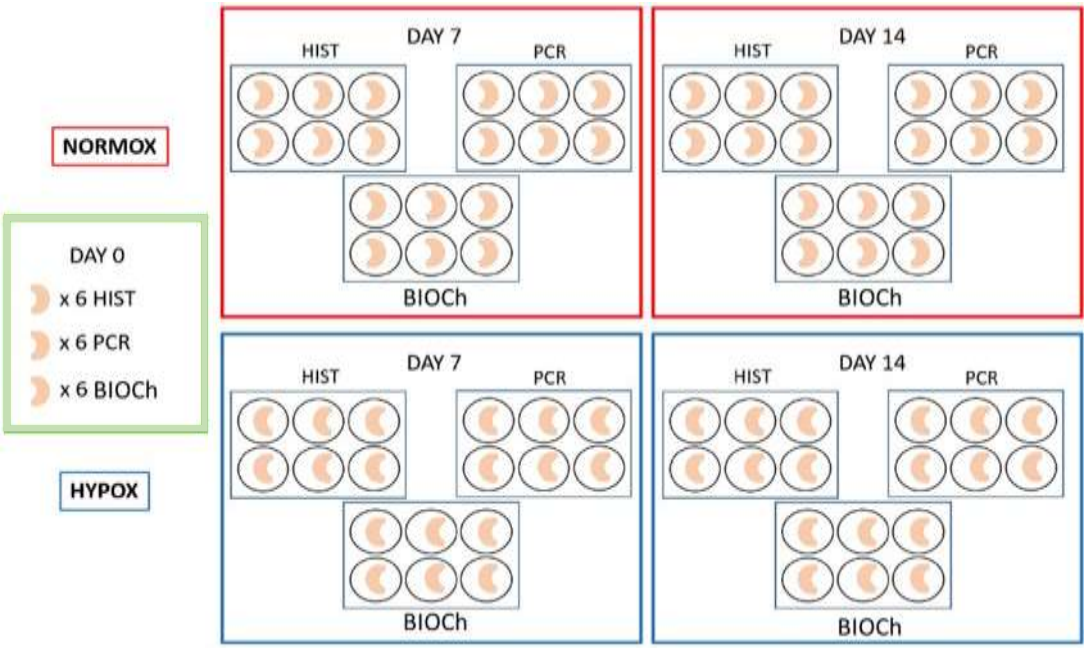

Fig 1 schematic drawing of the utilized protocol.

2.3 Biochemical analysis

For each experimental group (0 days, hypoxic and normoxic at 7 and 14 days), 6 specimens were processed (total n: 30). The samples for biochemical evaluation were digested in papain (Sigma-Aldrich, Milan, Italy) for 16–24 hrs at 60°C; the digestion solution was composed of 125 µg/ml of papain (Sigma-Aldrich) in 100 mM sodium phosphate, 10 mM sodium EDTA (Sigma-Aldrich), 10 mM cysteine hydrochloride (Sigma-Aldrich), 5 mM EDTA adjusted to pH 6.5 and brought to 100 ml of solution with distilled water. The digested samples were stored at -80°C until analyses. Aliquots of the papain digests were assayed separately for proteoglycan and DNA contents. Proteoglycan content was estimated by quantifying the amount of sulphated glycosaminoglycans using the 1,9-dimethylmethylene (DMB) blue dye binding assay (Polysciences Inc., Washington, PA, USA) and a microplate reader (wavelength: 540 nm). The standard curve for the analysis was generated using bovine trachea chondroitin sulphate A (Sigma-Aldrich). DNA content was evaluated with the Quant-iT Picogreen dsDNA Assay Kit (Molecular Probes, Inc., Eugene, OR, USA) and a fluorescence microplate reader and standard fluorescein wavelengths (excitation 485 nm, emission 538 nm, cut-off 530 nm). The standard curve for the analysis was generated using bacteriophage lambda DNA supplied with the kit.

2.4 Real-time PCR assay

Tissues were homogenized and extracted by using RNeasy Mini Kit (Qiagen). Quantity and quality of RNA were determined by using Nanodrop 8000 (ThermoFisher Scientific). RNA was reverse transcribed by

using ImProm II reverse Transcription System (Promega). Amplification of cDNA was performed by using PowerUp SYBR master mix (ThermoFisher Scientific) on 7500 Fast Realtime PCR System (Applied Biosystems). The sequences of primers were listed on Table 1. The reactions were performed in three stages: holding stage initializing at 50°C for 20 s, then 95°C for 10 min; cycling stage at 95°C for 15 s, then 60°C for 1 m. The cycling stage was repeated for 40 cycles. Finally in the melt curve stage the reactions were set at 95°C for 15 s, followed by 60°C for 1 m, 95°C for 30 s and 60°C for 15 s. Data was analyzed according to comparative method [Livak and Schmittgen, 2001] where data were presented as fold change ($2^{-\Delta\Delta Ct}$ value) with **ΔCt = Ct (gene of interest) - Ct (beta actin) and ΔΔCt = ΔCt at day n - ΔCt at day 0, n = number of days of differentiation.**

No.	Gene	Sequence (5'-3')	Amplicon size (bp)	Reference
1	Beta actin F	CAAGGAGAAGCTCTGCTACG	245	Kreinest et al., 2016
	Beta actin R	AGAGGTCCTTCCTGATGTCC		
2	Collagen type IA1 F	CCAACAAGGCCAAGAAGAAG	64	Kreinest et al., 2016
	Collagen type IA1R	ATGGTACCTGAGGCCGTTCT		
3	Collagen type II F	CACGGATGGTCCCAAAGG	102	Kreinest et al., 2016
	Collagen type II R	ATACCAGCAGCTCCCCTCT		
4	Sox9 F	CCGGTGCGCGTCAAC	119	Kreinest et al., 2016
	Sox9 R	TGCAGGTGCGGGTACTGAT		
5	HIF1alpha F	AGGAATTATTTAGCATGTAGACTGCTGG	73	Gelse et al., 2008
	HIF1 alpha R	CATAACTGGTCAGCTGTGGTAATCC		

Tab. 1 Primer sequences, F: Forward, R: Reverse

2.5 Statistical analysis

Statistical analysis was performed with SAS statistical software (ver. 9.3, Cary, NC, USA). Data from the biochemical and RT-PCR analyses were analysed using 2-way ANOVA with time (0, 7 and 14 days) and treatment (normoxia or hypoxia) as main factors. The individual meniscus was considered as the experimental unit. The data are presented as least-square means (LSM) with standard errors. Differences between means were considered significant at $p<0.05$.

3. Results

3.1 Morphological evaluation (Histochemistry and immunofluorescence)

Menisci were analysed via histochemical staining to evaluate matrix deposition and general morphology of cells in each time point and under the two different oxygen tensions (Fig. 2).

Initially, neonatal meniscus presents a fibroblast-like phenotype with some degree of matrix deposition even if limited to the nuclei (Fig. 2A, white arrowhead). After seven days in culture (T1; Fig. 2B and 2D), a

round shape of the cells is achieved mirroring a fibro-chondrocytes like phenotypization, both under normoxic (Fig. 2B, white arrow) and hypoxic (Fig. 2D, white arrow) conditions, however matrix production is maintained only in the hypoxic one (Fig. 2D, asterisk), mainly intranuclear and to a lesser extent also extracellular.

The last experimental point (T2; Fig. 2C and 2E) is characterized by a higher production of matrix, mainly in the extracellular space, only in the samples cultured under hypoxic conditions (Fig. 2E, asterisk), while the normoxic ones present pattern similar to the previously showed in T1, with round-shaped cells but no production of extracellular matrix (Fig. 2C).

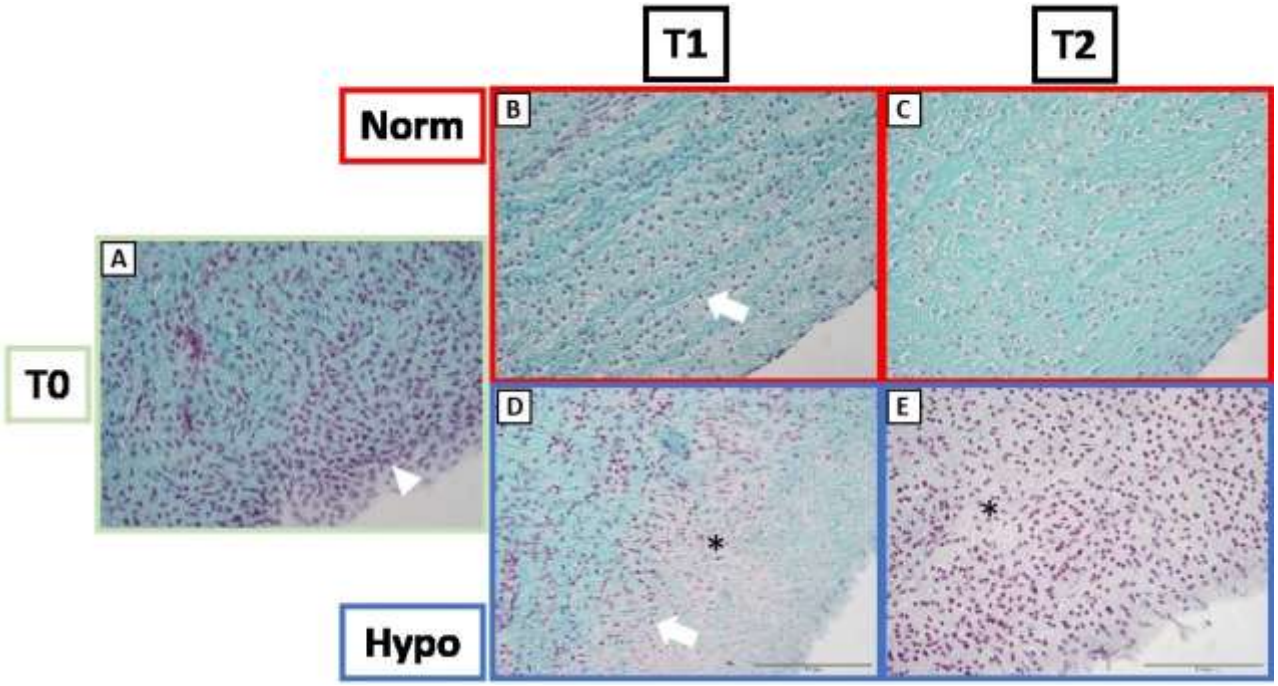

Fig. 2 Safranin-O staining of native meniscus (T0; A) and after 7 (T1; B, D) and 14 (T2; D, E) days of differentiated O_2 culture conditions. B, C: normoxic culture (21% O_2); D, E: Hypoxic culture (1% O_2). Arrowhead: fibroblast-like cells; arrow: Fibro-chondrocyte-like cell; asterisk: matrix deposition.

Histochemical results seem to be confirmed by the double immunofluorescence (Fig. 3).

The starting point is characterized by a tissue composed of elongated fusiform nuclei (Fig. 3A, blue) that express only Sox9 (Fig. 3B, green) protein, with no expression of collagen type II.

At seven days (T1) under both oxygen tension conditions, nuclei acquired a more rounded shape (respectively, Fig. 3D and 3G) and express both Sox9 (Fig. 3E and 3H, green) and collagen type II (Fig. 3E and 3H, red). Collagen type II expression is strictly linked to nuclei in the normoxic tissue (Fig. 3E, nuclear co-expression of collagen type II and Sox9, yellow), on the other hand, under hypoxic condition it present both an intranuclear (Fig. 3H, nuclear co-expression of collagen type II and Sox9, yellow) and a frankly extracellular expression (Fig. 3H, red).

At fourteen days, the last experimental time point (T2), two different patterns are showed. Tissues that underwent to normoxic culture are characterized by cells that seems to lose their round shape in favour

of a more elongated one (Fig. 3J, blue). Further, the expression of collagen type II is practically zeroed (Fig. 3K, red) and that of Sox9 is greatly diminished and, when present, it is limited to the (few) still rounded nuclei (Fig. 3J, green).

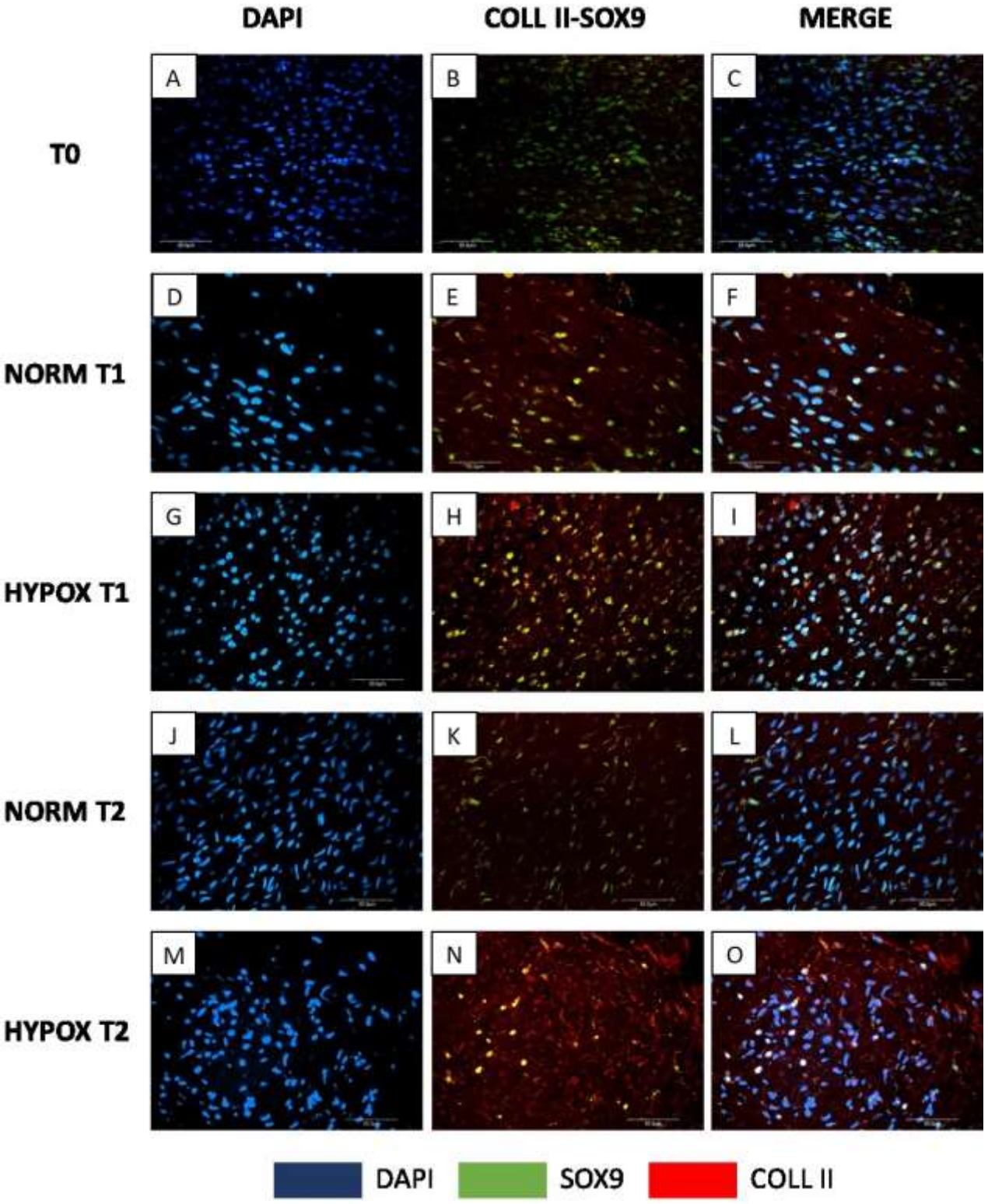

Fig. 3 Double immunofluorescence of meniscal samples. A-C: native meniscus; D-F: meniscus cultured under normoxia for 7 days (T1); G-I: meniscus cultured under hypoxia for 7 days (T1); J-L: meniscus cultured under normoxia for 14 days (T2); M-O: meniscus cultured under hypoxia for 14 days (T2). Blue: DAPI; green: SOX-9; red: Collagen type II; yellow: co-expression of SOX-9 and collagen type II.

On the contrary, hypoxic-cultured menisci present cells that still conserve their round shape nuclei (Fig. 3M) and a well-defined extracellular deposition of collagen II (Fig. 3N, red), not strictly linked to the expression of Sox9 (Fig. 3N, green or yellow when co-expressed).

3.2 Biochemical analysis

No statistical differences are showed regarding GAGs production by biochemical evaluation (Fig. 4A). However, some differences are described for DNA and GAGs/DNA ratio. DNA (Fig. 4B) quantification reflects the number of cells: T0 showed the higher cellularity respect to the other timepoints ($p<0,05$, with all comparisons except T1 under normoxia). At T1, under normoxia the cellularity is midway between T0 and T1 under Hypoxia ($p>0,05$ for the both comparisons), while T1 under hypoxia show a significant difference with the T0 ($p<0,05$).

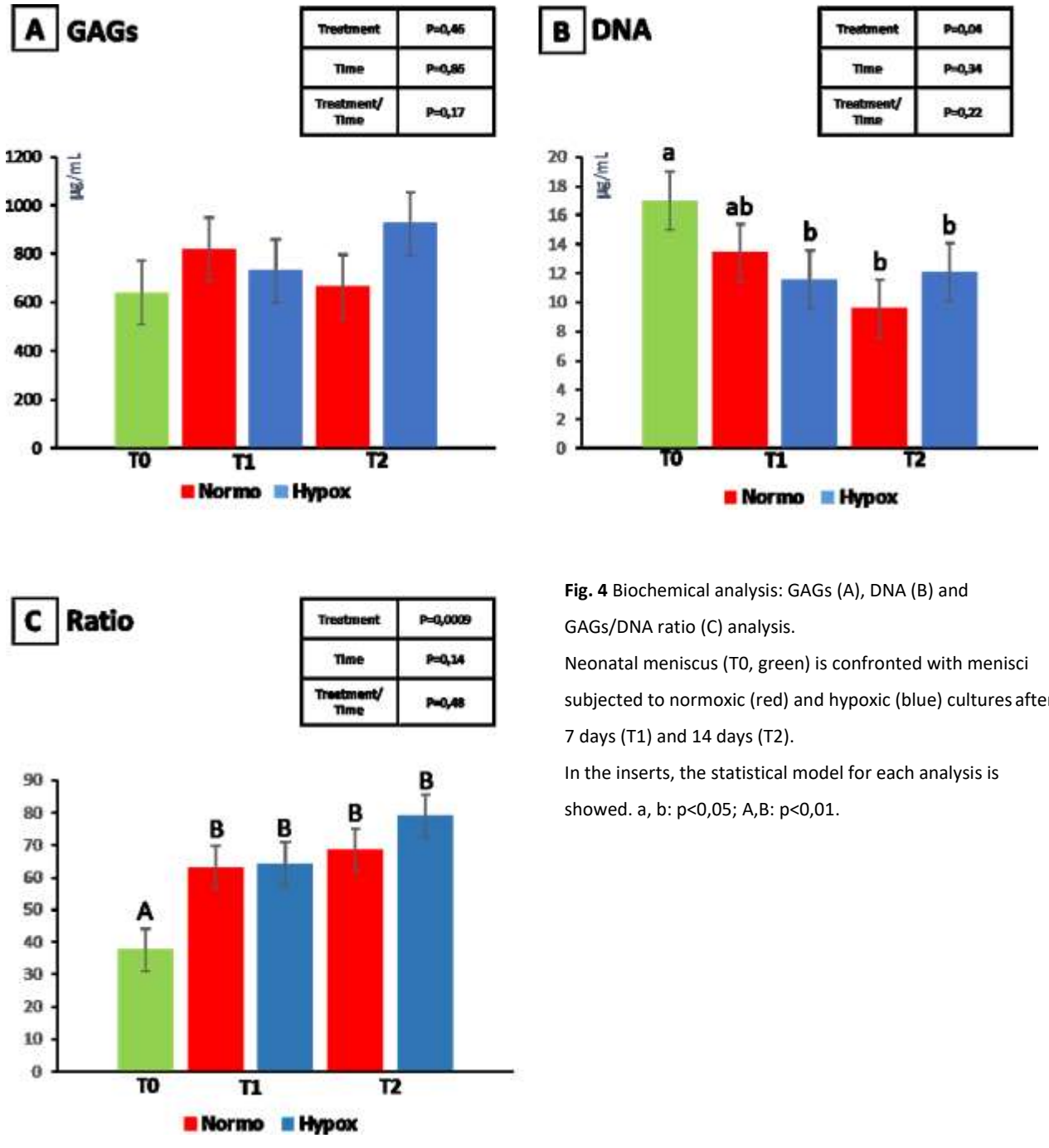

Fig. 4 Biochemical analysis: GAGs (A), DNA (B) and GAGs/DNA ratio (C) analysis.
Neonatal meniscus (T0, green) is confronted with menisci subjected to normoxic (red) and hypoxic (blue) cultures after 7 days (T1) and 14 days (T2).
In the inserts, the statistical model for each analysis is showed. a, b: $p<0,05$; A,B: $p<0,01$.

However, at T2 the number of cells is quite the same for both the conditions, with no difference respect to the previous time point under hypoxia and a significant difference with T0 and T1 under normoxia. The ratio between GAGs production and cells (GAGs/DNA; Fig. 4C) reflects the grade of maturation achieved by the tissue, since a mature tissue is characterized by a small number of cells that produce a high amount of matrix: higher is the value of the ratio, higher is the degree of maturation achieved by the tissue. It is possible to see how T0 corresponds to the lowest value for this parameter, characterized by a high number of cells that produce a little quantity of GAGs.
However, all the other comparisons show a higher ratio respect to the T0 ($p<0,01$), but no differences between the different experimental points.

3.3 Real-time PCR assay

To evaluate the effect of the two oxygen tensions, different genes linked to the maturation process of meniscal tissue and to the adaptive response to hypoxia were evaluated.
Collagen type I (Fig. 5A) is mainly up-regulated in the normoxia cultured menisci both at seven ($p<0,05$) and fourteen ($p<0,01$) days respect to those underwent to hypoxia.
Hypoxia induced factor- 1α (HIF-1α; Fig. 5B) is, as it may be expected, more expressed in the hypoxic meniscus at seven days of culture ($p<0,05$), however, interestingly no statistically significant differences are showed in the later time point, even if with a slightly higher value presented in the hypoxic one.
Sox9 (Fig. 5C) genes are highly up-regulated in the hypoxic meniscus after seven days of culture ($p<0,05$) respect to the normoxic one. No differences are reported at 14 days of culture under the different oxygenation conditions for this parameter. Finally, collagen type II (Fig. 5D), expression of meniscal tissue maturation, is highly up-regulated in the meniscus after 14 days of hypoxic culture ($p<0,05$). Furthermore, a time effect is present when it is considered the quantification of Hif-1a and collagen type II genes (for both the differences between T1 and T2, $p<0,01$).

4. Discussion

Oxygen delivery is essential in the first phase of meniscus' development since the absence of gait loading and movement does not allow the correct distribution of nutrients from synovial fluid to meniscal cells [Gray, 1999]. Since the full bearing of the joint is achieved, some modifications in the vascularization, the innervation and the cellular density start [Gray, 1999; Di Giancamillo et al., 2017]. These modifications are strictly linked to the achievement of the regionalization of the cellular phenotypes that characterized mature meniscus: with fibroblast-like cells scattered all over the outer zone and fibro-chondrocyte-like cells concentrated in the inner zone [Makris et al., 2011].

During growth, vascularization, innervation and cellular density decrease until only the outer third of the tissue preserve the original grade of vascularization and innervation, while the innermost zone is practically avascular and devoid of innervation [Gray, 1999; Di Giancamillo et al., 2017]. Cells seem to adapt to this process of devascularization thanks to Hif-1alpha growth factor that seems to mediate the transformation of fibroblast-like cells into fibro-chondrocyte-like cells (the dominant phenotype of the innermost area of the meniscus), which are predisposed to survive to hypoxic environment (Fox et al., 2012; Makris et al., 2011).

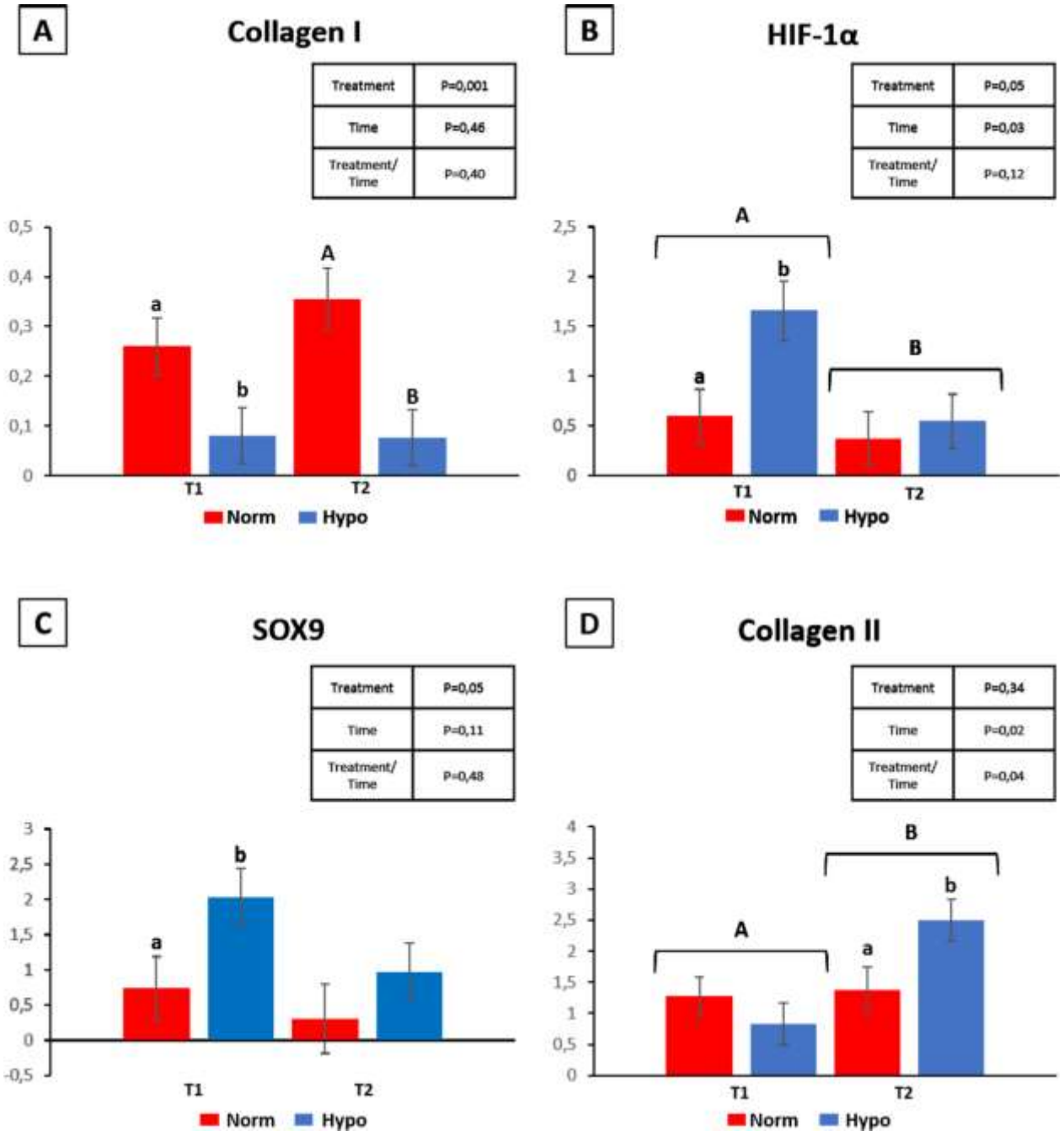

Fig 5 RT-PCR results of menisci after 7 (T1) and 14 (T2) days of normoxic (red) and hypoxic (blue) cultures. The expressions of Collagen type I (A), HIF-1α (B), SOX9 (C) and Collagen type II (D) were investigated. Results are reported as down-regulation or up-regulation respect to the neonatal menisci (T0) and expressed like ratio. Inserts present the statistical model for each analysis. A,B: $p<0,01$; a,b: $p<0,05$.

In this study we tried to replicate this process *in vitro* by means of a hypoxic chamber, a specific device that allows to control the oxygen tension present in the tissue culture environment.
Histochemical results show an increase in the GAGs production after 7 and 14 days of hypoxic culture, respect to the normoxic control, associated to the maintained mature-like meniscal phenotype (characterized by the expression of GAGs and the presence of fibro-chondrocyte-like cells) even in the later experimental point.
Moreover, the expression of SOX-9, a marker of precocious chondrogenic phenotypical differentiation, and collagen type II, the final product of meniscal cells maturation, increase under the hypoxic stimulus starting from 7 days of culture (characterized by the intranuclear co-expression of SOX-9 and collagen type II) up to the well distributed extracellular deposition of collagen type II after 14 days.
On the other hand, menisci subjected to normoxic culture lose their mature phenotype and de-dedifferentiate to fibroblast-like cells, as it is also described in cell aggregate [Abesida et al., 2007].
These apparent changes in GAGs production under the two different environmental condition are actually not confirmed by quantitative methods, that show no significant statistical differences among the two treatments for this parameter. Thus, the positive role of hypoxia on the grade of maturation of the meniscal tissue seems to be effective primarily on the collagen network since, even if morphological differences are showed in qualitative analysis, interestingly, no differences are showed in the quantity of GAGs and in the GAGs/DNA ratio between hypoxic and normoxic cultured menisci.
Similar apparent discrepancies between histological and biochemical results were found by Abesida et al. (2006) in human cells aggregates subjected to the action of Fibroblast growth factor-2 (FGF-2) under both culture conditions. GAGs are strictly related to the capacity of meniscus to withstand compressive loads, thus, if the lack of any significant differences between hypoxia- and normoxia-exposed samples may be linked to the absence of the application of any kind of biomechanical forces upon the neonatal menisci is matter of interest and should be left to further studies.
Some Authors state that hypoxia is a more powerful stimulus than dynamic compressive loading [as reviewed by Pappata et al., 2019] in promoting chondrogenesis in human cartilaginous cells, however, this is not completely clear in our study concerning meniscus.
Furthermore, the role of the time on the production of GAGs may not be excluded *a priori* and the utilization of a more extended time points may clarify these findings.
Hypoxia may mimic the effect of biomechanics compression upon vessels described during the physiological development of the tissue after the start of full load-bearing gait [Gray, 1999] and thus induces the morphological adaptations that characterize the mature meniscus, starting from the expression of collagen type II and, at a later time (not reached in the present study), the production of GAGs. It is also noticeable that in this study were utilised neonatal cells, which were, of course, committed

to become fibro-chondrocytes but were also completely immature at the beginning of the study; this may have a role in the temporal distribution of GAGs in the tissue.

RT-PCR analysis allows to study and evaluate the effect of time on the production of the different meniscal proteins and make possible to follow chronologically the track that hypoxia has traced to the maturation. Initially, the low tension of oxygen impedes the degradation of Hif-1α by its specific hydroxylases, this event leads to the up-regulation of Sox9 genes at 7 days [Adesida et al., 2006; Adesida et al., 2007; Gunja and Athanasiou, 2010] and, consequently, of those of collagen type II at 14 days [as reviewed by Gunja and Athanasiou, (2010) and Pattappa et al. (2019)]. These genomic changes lead later to an effective increment of collagen type II. This increment, associated to the morphologic evaluation that described an even more round shape, typical of fibro-chondrocytes, of the cells under hypoxia, represents a recognized feature of meniscal maturation.

Noteworthy, Hif-1α gene expression decreases in 14 days tissue. Physiologically, meniscal and cartilaginous cells that reached the complete maturation reside in a hypoxic environment [as reviewed by Abesida et al. 2007] surrounded by extracellular matrix that isolates them to each other [as reviewed by Abesida et al. (2012) and Hellio Le Graverand et al (2001)], thus, they may present a higher resistance to hypoxia respect to the younger and highly vascularized ones and a minor dependence on Hif-1α adaptive effect. This may explain the decrease of Hif-1α observed in the last time point of this study. Moreover, Abesida et al. (2007), demonstrated how mature meniscal cells harvested from the outer region of the meniscus, and so less used to hypoxia, respond to this stimulus more than those originated in the inner and avascular part. In the present study this differentiation between inner and outer zones of the meniscus were not considered due to the described absence of regionalization in the initial stage of development [Makris et al., 2011], however, an effect of this feature on the described results may not be excluded. Furthermore, species-specific differences in the composition of the native menisci may also have a role in these findings (as previously described, pigs are able to walk since the first day after birth, differently to what happens in humans [Gray, 1999] and other species, such as dogs [Greer, 2014]).

However, Hif-1α pathway is not the only one that result into chondrogenic differentiation [Tan et al. 2011]: other Authors described the action of different growth factors (such as FGF-2, bFGF, TGF-b3 and IGF-1), that may be unlinked to the expression of Hif-1a [Abesida et al. 2012; Abesida et al. 2006; Liang et al. 2018].

Thus, the alternative effects, upon meniscal cells in the later stages of development, of a hypoxia-linked unconsidered endogenously produced growth factor (instead of the Hif-1α) should be evaluated and investigated in further studies.

In the present study, hypoxia seems to preserve the phenotypical characteristics of a mature tissue avoiding the typical de-differentiation that is observed in cellular culture. De-differentiation features, characterized by the loss of the fibro-chondrocytic round shape and of collagen type II production in

favour of a fibroblast-like elongated shape and the expression of only collagen type I, are found in this study in those tissue subjected to normoxic culture, starting from 7 days and completely at 14 days.

Even if similar positive results of the application of hypoxia are showed in literature in both aggregate meniscal cells [Abesida et al., 2006, 2007 and 2012] and cartilaginous samples [Pattapa et al., 2019], to our knowledge, this is the first study that evaluates the effect of hypoxia on the whole meniscal tissue, in particular in a neonatal one: in this way it has made possible to study the pure effect of hypoxia upon a committed cells population within its native extracellular matrix, without the application of any other stimuli.

Taken together the results of this study suggest a positive role of the hypoxia in the differentiation process of meniscal tissue, in particular, hypoxia seems to act as a booster for the production of the phenotypically mature matrix (triggering the SOX9-mediated production of collagen type II) and allow to maintain the mature phenotype that characterized the functional meniscus. However, the same positive effects seem to not affect the production of GAGs and thus an additive external stimulus (biomechanics?) to achieve the complete functional maturation of both meniscal cells and matrix should be considered.

5. Conclusion

This study shows that hypoxia can be considered as a booster to achieve meniscal cells maturation and opens considerably opportunities in the field of meniscus tissue engineering.

However, to achieve a complete maturation that consider both collagen network and GAGs production, the application of hypoxia alone may be not enough (at least considered a maximal culture time of 14 days) and the application of other stimuli (such as growth factors or biomechanics) seems to be still necessary.

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5. Discussion and Conclusions

In this thesis the effects of different, exogenous and endogenous, factors upon the development of meniscus are investigated.

The basis of tissue engineering is the full-knowledge of the anatomic structure of the tissue that is matter of interest and of all the modifications to which it is subjected under different stimuli.

In the first part of this thesis the morpho-functional changes that occur during growth were investigated.

Two aspects were investigated, the effect of growth on matrix and on the cells' phenotypes.

An increase in the matrix composition characterize the meniscus moving from the neonatal to the adult stage: increments in aggrecan and decorin productions and in the grade of organization of the collagen fibres (that are finally composed of both collagen type I and II in the mature meniscus and not of only collagen type I, like in the initial stages), were described during maturation.

These modifications depending on the phenotypical transformation of cells that are fibroblast-like (fusiform in shape, collagen type I producer and weak producer of GAGs) at the beginning into the typical fibro-chondrocyte-like cells (rounded in shape and producing both collagen type I and II, in addition to a higher and functional GAGs production) at the later stage.

These changes are mirror of the full biomechanical properties gained by the mature meniscus and reflect the progressive hyper-specialization that characterize this multifaceted structure.

Biomechanical stimulation seems to be the *fil rouge* that lead to the maturation.

In fact, at complete maturation under physiologic conditions, meniscus is characterized by two different main patterns that reflect its biphasic nature: the femoral and the tibial surfaces.

The disposition of the fibres and the composition of the matrix are strictly linked to the different biomechanical stimulations (compression and traction) to which the two portions are subjected.

In the second part of this thesis, it is showed how the application of this kind of stimuli should be controlled to direct the maturation in the proper way.

It was described as compression alone leads to an early maturation of the matrix (characterized by the production of GAGs and collagen type II) at the expense of a proper organization of the collagen fibres, leaving space to the hypothesis that tensile forces are essential to provide the correct organization of the collagen fibres.

On the other hand, even if hypoxia acts like a booster for the maturation process, the lack of any kind of biomechanical stimulation leads to the presence of a characteristic phenotypically mature cells that produce collagen type II, but not the proper rich-of-GAGs matrix.

In order to achieve the proper balance between cells and matrix, the perfect association of the different stimuli should be considered.

This thesis suggests how to choose the perfect solution (biomechanical or environmental stimulation) to achieve the desired maturation aspect of a hypothetical tissue engineered meniscal construct.

6. Future perspectives

Biomechanical stimuli are fundamental for ECM maturation and cell differentiation. Tissue engineered scaffolds, that hypothetically reproduce the biphasic nature of this tissue and are seeded with meniscal mature or immature meniscal cells, that are subdued to a combination of compressive and tensile forces may help in the proper maintenance of the phenotypical traits, in the first case, and to replicate the meniscal maturation as it occurs in nature, in the latter. In addition, the regulation of the culture environment to reproduce the natural environment in which meniscus use to reside may boost the phenotypic changes that characterised the functional cells and allow to maintain the differentiated and functional phenotypes that are essential to achieve the complete substitution of the damaged tissue.

The creation of a bioreactor that consider all these points may favoured the large-scale fabrication of meniscal substitutes and should be applied even in other field of the tissue engineering.

7. Supplementary works

Polito, U, Modina, SC, Di Giancamillo, A, Nguyen, VT, and Peretti, GM. (2019) Decorin age related variations in the distribution of pig extracellular matrix meniscus. J Biol Regul Homeost Agents. Mar-Apr; 33 (2 Suppl. 1):119 124.

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Andreis, ME, **Polito, U**, Modina, SC, Carnevale, L, Veronesi, MC, Di Giancamillo, A, Roccabianca, P, Di Giancamillo, *M. Quadriceps contracture induces hind limb ossification centres hypoplasia and deformation: radiographic and computed tomographic study in 13 dobermann pinscher littermates*.