Characterization of articular chondrocytes isolated from 211 osteoarthritic patients

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Introduction

Osteoarthritis (OA) is a highly disabling pathology which is worldwide investigated by the scientific community due to its increasing diffusion. This age-dependent degenerative disease induces the progressive damage of articular cartilage and subchondral bone and can eventually lead to the complete loss of joint functionality (Aigner et al. 2004; van der Kraan and van den Berg 2008). The combination of pharmacological and non-pharmacological therapies has been proved to be useful for the treatment of early- and middle-stage OA patients, but a successful clinical outcome is not granted when treating late-stage OA patients (Zhang et al. 2008). In this category of patients, the severe pain and the functional limitations caused by advanced OA degeneration are currently resolved mainly by joint replacement surgery.

In the last two decades, "advanced therapies" have been intensely investigated in developed countries, with the aim to find a biological solution to several degenerative pathologies. In cartilage tissue engineering, a possible strategy for an autologous approach would be to isolate chondrocytes from a cartilage biopsy, to expand the cells and possibly cryopreserve them for future re-intervention. Expanded chondrocytes are then seeded on a biomaterial and the cellularized construct is finally implanted back to the patient (Roseti et al. 2011). Cartilage tissue engineering applied to OA patients appears to be in contrast with the controversial opinion that healthy autologous chondrocytes or mesenchymal stem cells are necessary to obtain healthy cartilage in vitro (Tran-Khanh et al. 2005; van der Kraan and van den Berg 2008; Wang et al. 2006). Recent works, however, have demonstrated the possibility to use human OA chondrocytes to obtain autologous engineered cartilage with properties similar to those of the constructs obtained from non-OA chondrocytes (Carossino et al. 2007; Cavallo et al. 2010; Dehne et al. 2009; Tallheden et al. 2005). Moreover, a recent work (Neri et al. 2011) on human OA articular chondrocytes characterized their genetic stability during long term expansion, and demonstrated the safety of this cell type for autologous advanced therapies. Since the quality of cells used to generate the engineered tissue greatly influences the outcome, a characterization of OA chondrocytes in a wide patient group can help in extending the validity of the reported results.

In this work we analyzed specific features of chondrocytes collected from 211 osteoarthritic patients undergoing total joint replacement. The characteristics investigated were cellular yield and cell doubling rates, and the dependence between these parameters and some patients-related data.

Materials and methods

Chondrocyte isolation, expansion and data collection

Samples of adult human articular cartilage were harvested from subjects undergoing hip and knee routine arthroplasty procedures. With the patient's informed consent and in respect to the privacy law, the patient age, gender and joint type were recorded. Based on evaluation by orthopaedic surgeons, random knee samples were also graded from scores 1 to 4 using the ICRS protocol (Kleemann et al. 2005), where higher scores indicate higher levels of cartilage degeneration. Minced cartilage fragments (2–3 mm^2) were weighted and chondrocyte isolation was carried out by cartilage digestion in 0.15 % collagenase type II (Worthington) performed for 22 h at 37 °C, as previously described (Jakob et al. 2003).

Viable cells were counted by trypan blue dye exclusion. Freshly isolated chondrocytes (P0) were plated for expansion at a density of 1×10^4 cells/cm^2 for the first passage (P1) and cultured with complete proliferation medium (CM) containing Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 % fetal bovine serum (FBS, Lonza), 1 mM sodium pyruvate, 100 mM HEPES buffer, 100 U/ml penicillin, 100 µg/ml streptomycin, 0.29 mg/ml L-glutamine, 1 ng/ml TGFb1, 5 ng/ml FGF-2 (PeproTech) (Barbero et al. 2003). Where not otherwise specified, Invitrogen products were used. For the second passage (P2) cells were plated at a density of 0.5×10^4 cells/cm^2. Cell doubling rate (*d*) was calculated as

$$\mathrm{d} = \frac{log_2\left(\frac{N}{N_0}\right)}{\mathrm{t}} \quad (1)$$

where N and N_0 are the final and initial cell number, respectively, and t is the expansion time. The cellular yield was calculated as the ratio between the viable cell number at P0, expressed in millions of cells, and

	Males [%]	Females [%]
Hip patients	42%	58%
Knee patients	12%	88%
Total patients	31%	69%

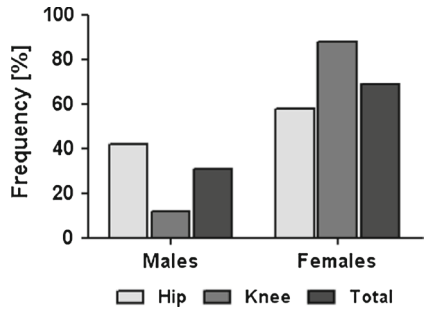

Fig. 1 Frequency distribution of patient gender. The patients, all affected by osteoarthritis, were subjected to total hip or knee replacement

the wet weight of the cartilage fragments, expressed in grams.

Data processing and statistical analysis

The software Graph Pad Prism® was used to order and process the data. For each patient, the available data were joint type, patient age, patient gender, ICRS grading, cellular yield, doubling rate at P1 and at P2. Mean and standard deviation were calculated for each parameter and relative normalized frequency (2) histograms were obtained. The normalized frequency was calculated as

$$nf = \frac{f}{S_c \times N_s} \tag{2}$$

where f is the frequency of the data inside the considered class, S_c is the spacing of the class and N_s is the sample size (132 patients for the hip group, 88 patients for the knee group). Relative normalized frequency histograms were also obtained for the scored knee cartilage samples. For all the relative normalized frequency histograms the Poisson distribution was calculated.

Non normal distribution of data was determined with a D'Agostino & Pearson normality test. Correlation between patients' age, cellular yield, and proliferation rate at P1 and at P2 was determined calculating the Spearman coefficient r and considered significant for $p < 0.05$. To compare independent groups, Kruskal–Wallis test followed by Dunn's multiple comparison test was performed and differences were considered significant for $p < 0.05$.

To better evaluate age-related differences in cellular yield and proliferation rate, patients were grouped in the following age classes: ≤60, 65–70, ≥75 years, composed of the same number of patients (n = 47). Four years gaps between classes were left to avoid overlapping.

Box-plots were used for the visualization of the main parameters of the data statistical distribution both for age classes and for ICRS score groups (minimum, 25th percentile, 75th percentile, maximum).

Results

Patients' gender distribution showed that 42 % of hip arthroplasties were performed on males and 58 % on females (Fig. 1). For knee arthroplasties the percentage of male versus female patients was 12 % versus 88 %. Considering both arthroplasty types together, female patients were 69 % of the total. Patients' age ranged between 30 and 90 years and the frequency histograms showed a mean age of 66 years, both for knee and hip patients (Fig. 2a–c). The ICRS grading system was applied to samples of knee cartilage randomly chosen among the donors, to classify the samples from scores 1 (low damage) to 4 (high damage). The mean age of patients did not differ significantly between scores and ranged from 65 to 69 years, as shown in frequency distribution graphs in Fig. 2d–g.

Cellular yield values ranged between 0.1 and 5.5 million cells/g of tissue, with a mean of 1.85 ± 1.37 and 1.62 ± 1.08 million cells/g of tissue for hip and knee samples, respectively (Fig. 3a–c). For the different ICRS scores, mean values of cellular yield were similar up to score 3 (about 1.4 million cells/g of tissue), whereas it was lower for highly degenerated tissue (score 4, 0.9 million cells/g of tissue).

The analysis of the correlation between patients' age, cellular yield and proliferation rate at P1 and at P2

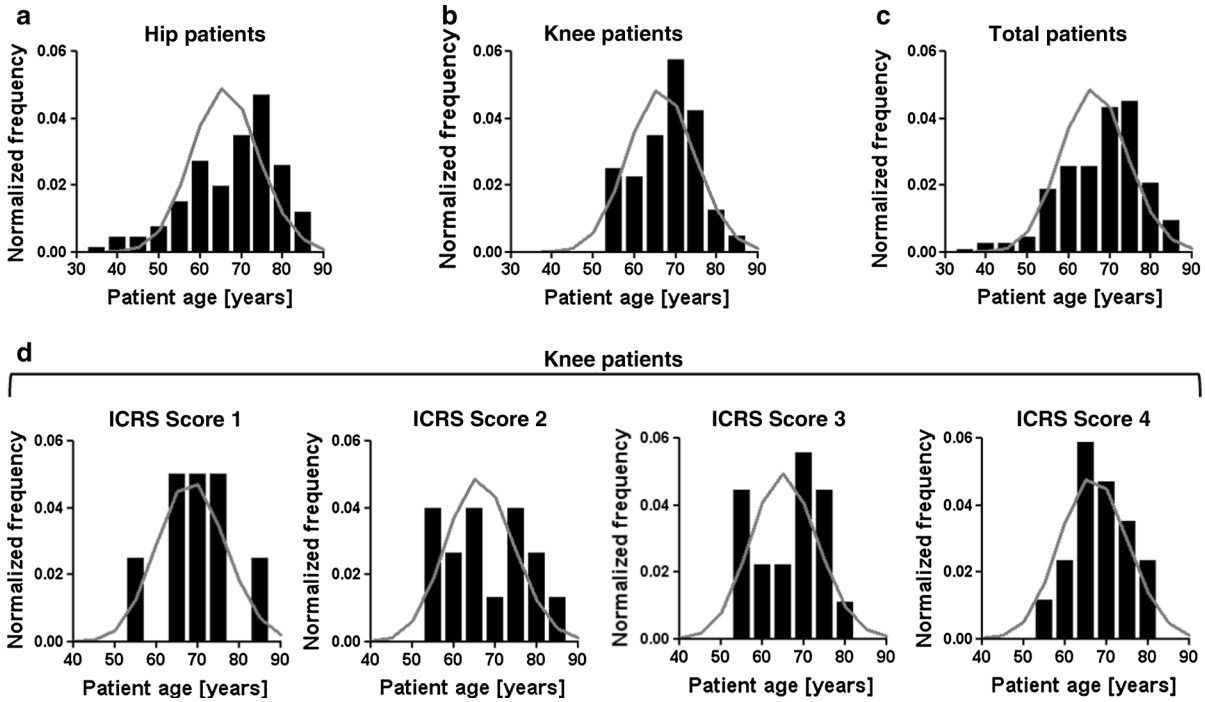

Fig. 2 Relative normalized frequency distribution obtained for increasing classes of patient age for the hip (**a**), knee (**b**), total (**c**) and increasing ICRS scores (**d**–**g**). The frequency histograms are approximated by Poisson's distribution

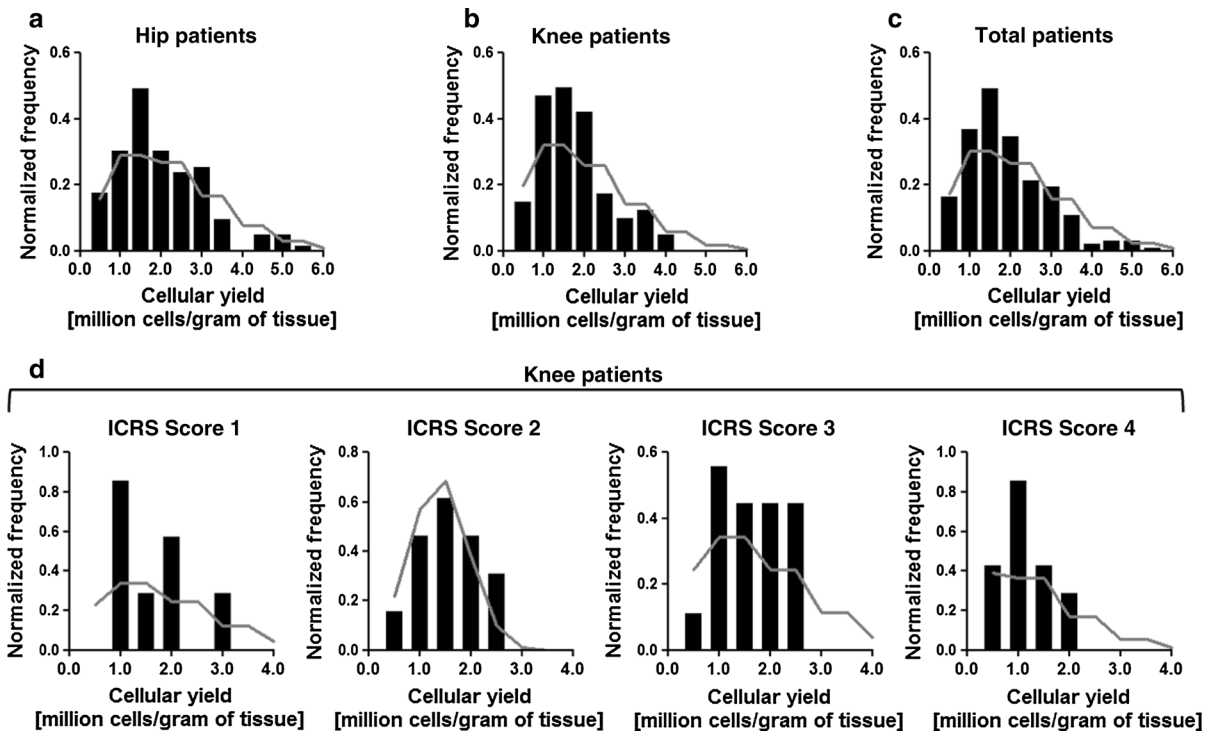

Fig. 3 Relative normalized frequency distribution obtained for increasing classes of cellular yield for the hip (**a**), knee (**b**), total (**c**) and increasing ICRS scores (**d**–**g**). The frequency histograms are approximated by Poisson's distribution

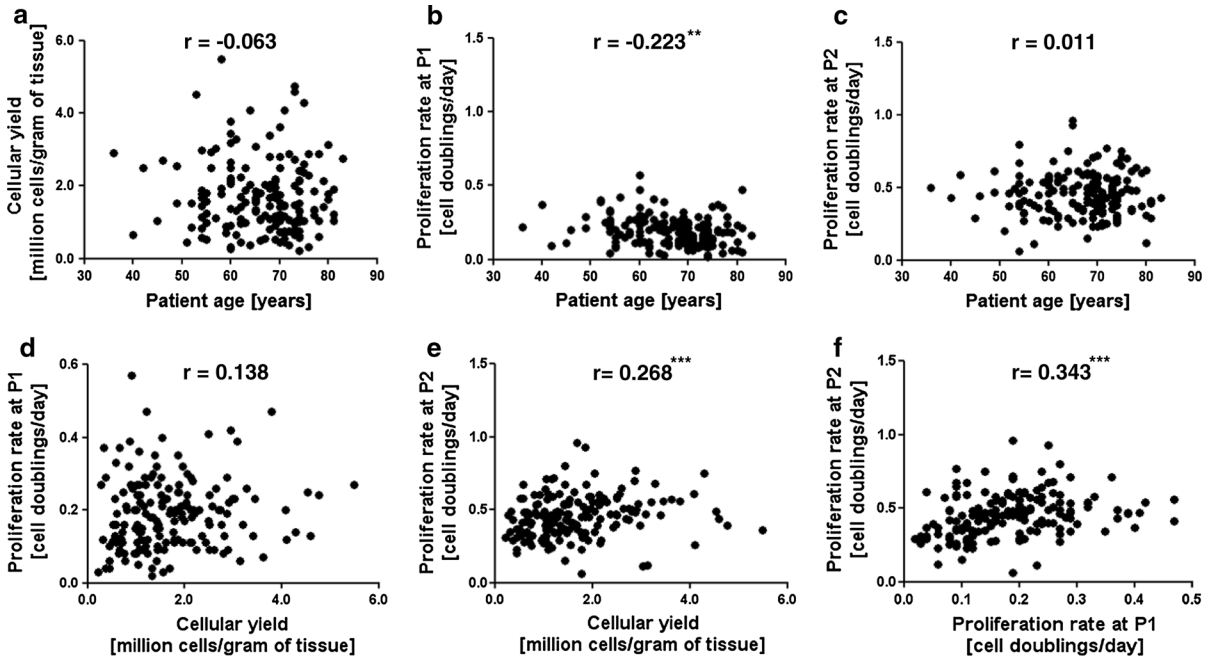

Fig. 4 Correlations among individual data groups. **a** Cellular yield versus patient age, **b** proliferation rate at P1 versus patient age, **c** proliferation rate at P2 versus patient age, **d** proliferation rate at P1 versus cellular yield, **e** proliferation rate at P2 versus cellular yield and **f** proliferation rate at P2 versus proliferation rate at P1. Correlation coefficients (Spearman r) are indicated in each graph. $^{**}p < 0.01$, $^{***}p < 0.001$

are reported in Fig. 4 with the corresponding correlation coefficients (Spearman r). Significant correlations were observed between age and doubling rate at P1 ($r = -0.223$, $p < 0.01$), showing a decrease of doubling rate at P1 with increasing age, and between doubling rate at P2 and at P1 ($r = 0.343$, $p < 0.001$), demonstrating a trend between cell growth at both passages. A significant correlation was found also between cellular yield and proliferation rate at P2 ($r = 0.268$, $p < 0.001$), suggesting that cells deriving from samples with a higher cellular yield, retain a greater proliferation potential during expansion.

Pair wise comparison of data, grouped on the basis of patient age classes, homogeneous in size, are shown in Fig. 5. The average cellular yield did not significantly differ between age classes (Fig. 5a). We found statistically significant differences on proliferation rates at P1 between the age class ≤60 as compared to

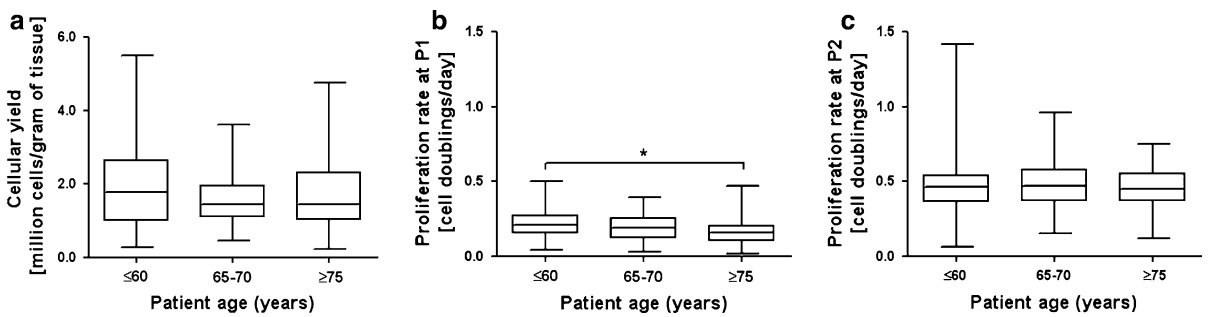

Fig. 5 *Box plots* showing pair-wise comparisons of grouped data pertaining to patient age and cellular yield classes homogeneous in size. **a** Cellular yield versus patient age, **b** proliferation rate at P1 versus patient age, **c** proliferation rate at P2 versus patient age. The values delimiting the *box* in the *box plots* are the 25th and 75th percentiles of the data distribution. $^{*}p < 0.05$

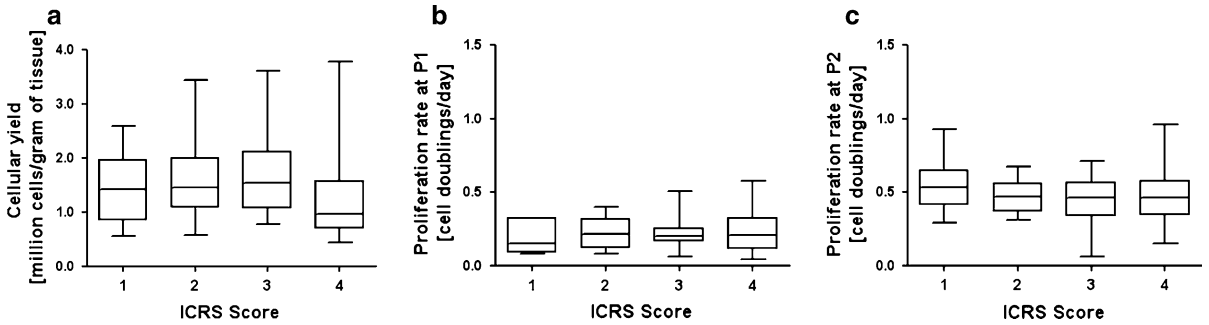

Fig. 6 *Box plots* (**a–c**) for the ICRS-scored knee cartilage samples. The values delimiting the *box* in the *box plots* are the 25th and 75th percentiles of the data distribution

the ≥75, which was characterized by the lowest proliferation rate (Fig. 5b). Proliferation rates at P2 were higher than at P1, independently from the patient age (Fig. 5c).

No significant correlation was found among ICRS scores, cellular yields and proliferation rates at P1 and P2, as shown in Fig. 6. However, as the cartilage damage level increased, the mean cellular yield slightly decreased, whereas the proliferation rates did not differ with increasing scores. As already observed for data on hip and knee population, proliferation rates at P2 were higher than the rates at P1.

Discussion

OA is fully recognized as an age-dependent degenerative disease (Loeser et al. 2012). Considering the high and increasing life expectancy in developed countries, osteoarthritis is assuming an important social effect. Coherently, countermeasures to OA such as cellular therapies are intensely investigated (Goldring and Goldring 2007). In this study, adult human articular chondrocytes were isolated from anatomical specimens discarded during total joint replacement, with the aim to characterize them in view of their potential use as source for autologous cell therapy. More than 200 OA patients were included in this analysis, which required, for our institution, a period of 4 years for data collection. Accordingly with literature (Cicuttini et al. 2003; Musumeci et al. 2011; Wluka et al. 2001) we found that the majority of patients subjected to total joint replacement were postmenopausal women. The age of patients in our analysis ranged from 30 to 90 years old, but the frequency distributions showed that the highest amount of patients belonged to the range 65–70 years. The pair-wise analysis of data on cellular yield and proliferation rates showed that the patient age is not statistically correlated to the cellular yield, as in (Bobacz et al. 2004), but was negatively correlated with the proliferation rate at P1, which followed a trend analogue to the proliferation rate at P2. As already reported in the literature (Barbero et al. 2004) for healthy chondrocytes, the most relevant reduction in cellular yield is observed between patients younger than 40 years and older patients, then with increasing age this feature remains almost constant. 75 % of our patients are older than 63 years: this could explain the lack of significant influence of patient age on cellular yield. In accordance with previously published data on healthy chondrocytes (Dozin et al. 2002), cell proliferation significantly decreased with increasing age at P1, but this significant correlation was not found between age and doubling rate at P2. A possible explanation can be found in the use of TGFβ1 and FGF2, growth factors able to increase the proliferation rate and to maintain the chondrocyte phenotype during the expansion procedures. This property is particularly useful when large amounts of cells are needed requiring a long-term expansion (Barbero et al. 2003). Thanks to the use of TGFβ1 and FGF-2, chondrocytes started soon to proliferate. The offset difference in cell proliferation rates that we found between P1 and P2 is in accordance with previous work (Barlic et al. 2008; Lin et al. 2006) and can be attributed to an initial lag phase where cells recover from isolation procedure. Interestingly, however, a relationship between the proliferation rate at P1 and at P2 was found in our data suggesting that cells with the lowest proliferation rate at P1 were also less proliferative at P2 and that the cells with high proliferation rate at P1 maintained this feature at P2.

To correlate analyzed cellular parameters with the grade of cartilage degeneration, we evaluated random samples scored on the basis of ICRS scale. A lower cellular yield was observed in score 4 specimens. This result can be attributed to the higher degeneration level of this tissue whereby the inflammatory state of the tissue can affect cellularity. The proliferation rates for samples with different scores are in accordance with previously reported results (Yin et al. 2011): score has a slight influence only on the initial phase of in vitro expansion, whereas cell proliferation during the subsequent passages was independent from the degeneration of the excised cartilage.

In conclusion, our study allowed a systematic characterization of basic parameters as cellular yield and proliferation rates of chondrocytes originating from a wide group of OA patients. This characterization could be useful in view of a possible autologous cell therapy approach for osteoarthritis, as it is fundamental to determine the quality of the cell source, known to greatly influence the outcome of engineered tissue.

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