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Donor age and breed determine mesenchymal stromal cell characteristics

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Abstract

Background Mesenchymal stromal cells (MSCs) hold significant potential for various applications in regenerative medicine and tissue engineering. Initially considered as a single cell type with defined characteristics, MSCs are now known as a heterogeneous cell population with remarkable differences in their properties. No consensus exists on how donor age affects MSC characteristics, like proliferation. Additionally, differences in differentiation capacities and immunophenotype could arise when MSCs are isolated from different animals breeds, which is relevant for experimental and preclinical studies of MSC-based treatments.

Methods In this study, we isolated bovine adipose tissue-derived MSCs from three age categories, i.e. fetal, calf, and adult, and of two different breeds, i.e. Holstein Friesian (HF) and Belgian Blue (BB). MSC characterization included tri-lineage differentiation, proliferation and senescence assays, and immunophenotyping using multi-color flow cytometry.

Results Especially fetal and calf HF-MSCs showed a high proliferation capacity, where 4 and 6 out of 7 donors, respectively, could surpass 30 population doublings. Adipogenic differentiation potential was higher for fetal and adult HF-MSCs. Furthermore, breed, but not age, affected their osteogenic differentiation potential, with BB-MSCs performing better. Evaluation of cell surface marker expression revealed a breed effect, as calf HF-MSCs showed a higher percentage of Cluster of Differentiation (CD)34$^+$ cells compared to calf BB-MSCs, which was correlated with both osteogenic differentiation and proliferation potential.

Conclusions Our findings clearly show the impact of donor characteristics such as age and breed on MSC proliferation, immunophenotype, and differentiation potential, illustrating the importance of selecting the appropriate MSC donor for MSC-based treatments when allogeneic MSCs are considered.

Keywords Donor, Bovine, Differentiation, Proliferation, Immunophenotype, Age, Breed, Mesenchymal stromal cells

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Introduction

Mesenchymal stromal cells (MSCs) are considered an interesting cell type for a multitude of potential applications in regenerative medicine and tissue engineering [1–3], due to their self-renewal, multi-lineage differentiation, and immunomodulatory properties [4–8]. Human MSCs are characterized according to guidelines formulated by the International Society for Cell & Gene Therapy (ISCT) [9, 10]. They should be: (i) plastic adherent under standard culture conditions, (ii) capable of differentiating towards the osteogenic, adipogenic and chondrogenic lineage, and (iii) express specific surface markers such as Cluster of Differentiation (CD)73, CD90 and CD105, but lack expression of hematopoietic or endothelial markers such as CD11b or CD14, CD34, CD45, CD79α or CD19 and Major Histocompatibility Complex (MHC) class II.

Although plastic adherence and tri-lineage differentiation are cross-species characteristics, the expression of cell surface markers is less uniform [11–13]. For example, the ISCT has stated that the lack of CD34 expression is not as definitive, as subpopulations of human adipose tissue (AT)-derived MSCs have been shown to express CD34 [14, 15]. Also in bovine MSCs isolated from subcutaneous AT a variable expression was observed for CD34 [12]. When these criteria were first proposed, MSCs were considered a single cell type with defined characteristics, while now they are known as a heterogeneous cell population with differences in their properties. In particular, donor-to-donor heterogeneity is considered to be the decisive factor in the observed differences in MSC characteristics and thus in treatment efficacy when clinically used [16, 17].

Although it is often assumed that increasing age is associated with reduced MSC proliferation, there is no consensus on how donor age affects MSC proliferation [18–23]. Indeed, multiple studies observed a decreased cellular proliferation capacity in older donors while morphological changes associated with senescence increased, i.e. transition from fibroblast-like to epithelial-like cells, which was further confirmed by high levels of intracellular β-galactosidase and reactive oxygen species [19, 21, 24–27]. Sanghani-Kerai et al. (2018), on the other hand, did not observe any effect of age on the proliferation potential of rat bone marrow (BM)-derived MSCs, with increasing donor age [28].

The effect of donor age on the MSC differentiation potential is even less defined. Regarding adipogenic differentiation, human dental pulp MSCs of adult donors (25–30 years) differentiated more efficiently to adipocytes compared to young donors (< 19 years) [23], while other studies observed a significant decrease in adipogenic differentiation potential with increasing donor age for human BM-MSCs and gingival-MSCs [26, 27]. On the other hand, osteogenic differentiation of human dental pulp MSCs was comparable between the age groups up to 30 years [23], but was significantly reduced in AT- and gingival-derived MSCs of donors older than 60 [27, 29]. Several studies showed that the chondrogenic differentiation potential of MSCs decreases with increasing age [18, 20, 29, 30]. In conclusion, there is no clear linear relationship between donor age and MSC differentiation capacity, although it must be mentioned that different species, age groups, and MSC tissue sources were used in these studies.

Regarding the immunophenotype, most studies did not observe differences in marker expression between MSCs from different age groups [24, 27, 28]. However, an increased expression of CD71, CD90, CD106, CD140b, CD146, CD166, and CD274 has been reported in younger human donors [31], while a reduced expression of CD73 in old mice [32] and increased expression of CD90 in elderly people has been observed [30].

Breed differences are also likely to affect the proliferation, differentiation capacity, and immunophenotype of MSCs, as reported for equine, canine, and porcine MSCs. BM-derived MSCs from different large dog breeds (namely Border collie, German shepherd, Labrador, Malinois, Golden retriever, and Hovawart) varied in their proliferation and tri-lineage differentiation potential [33]. The MSCs actively divided only for 4 weeks in culture, except for the MSCs of Border collies which divided for a longer time than cells from other dog breeds. The MSCs of different canine breeds underwent efficient osteogenic differentiation, however, the average calcium deposition was higher in the MSCs of border collies, golden retrievers, and labradors compared to other breeds. The MSCs of all breeds had the same adipogenic differentiation potential [33]. These variations in the differentiation potential of MSC were also observed in swine [17, 34, 35]. Son et al. (2021) reported that the osteogenic differentiation potential of dental pulp MSCs and periodontal ligament MSCs, collected from a male Yucatan miniature pig (9 months old), was inferior to that of a male domestic pig breed (six months old) [35]. More recently, Li et al. (2023) reported differences in osteogenesis-related genes between synovial membrane MSCs from Angeln Saddleback and German Landrace breeds. Higher levels of collagen 1A1, *NANOG* and osteopontin mRNA were observed in synovial-MSCs from Angeln Saddleback, while the expression of alkaline phosphatase and osteocalcin was lower when compared to German Landrace [17]. Furthermore, in horses, significant differences in MHC class II and CD90 expression were observed between the closely related Standardbred and Thoroughbred horses [36]. As cells from animal origin are frequently used in veterinary regenerative studies and as relevant model for translational studies, these breed effects should be considered.

The ability to control and/or predict the characteristics of MSCs, such as their proliferation and differentiation potential, is essential for their clinical use. Especially when allogeneic MSCs are considered, the impact of donor characteristics on MSC properties should be properly evaluated to select the most appropriate MSC donor. However, the influence of donor age and breed on MSC characteristics remains poorly defined. This knowledge gap limits the ability to select and utilize MSCs for experimental and therapeutic purposes. To study the impact of age and breed on MSC characteristics and provide comprehensive insights, we isolated MSCs from three age categories, i.e. fetal, calf, and adult, and in two distinctly different breeds, Holstein Friesian (HF) and Belgian Blue (BB), within one species, i.e. bovine, and one source, i.e. AT. The characteristics of MSCs were evaluated by trilineage differentiation assays, proliferation and senescence assays, and immunophenotyping using multi-color flow cytometry. We hypothesized that MSC isolated from younger donors will show enhanced proliferation and differentiation capacities, accompanied by a reduced rate of senescence. Additionally, breed-related genetic and metabolic differences might result in significant variations in MSC characteristics [33, 36, 37].

Materials and methods

Cell isolation methods

Bovine MSCs were isolated post-mortem from subcutaneous AT of healthy HF and BB cattle in three age groups ($n = 7$ per breed × age group), including fetal (5–8 gestational months; both sexes), calf (6–11 months; males), and adult (≥ 2 years old; females) (Table S1). Fetal gestational ages were calculated by measuring the crown-rump length, commonly used to determine fetal age [38].

Using an enzymatic digestion method, bovine MSCs were isolated from subcutaneous AT, as previously described [12, 39]. Briefly, AT was collected in the local abattoir and transported within 1–2 h to the lab. After extensive washing, the tissue was dissected into smaller pieces of approximately 1 mm [3], weighed, and digested in a 1 mg/mL Liberase™ solution (Sigma) for 6 h at 38.5 °C in a humidified atmosphere containing 5% CO. Subsequently, the enzymatic reaction was neutralized with an equal amount of culture medium, consisting of low glucose Dulbecco's Modified Eagle Medium (LG-DMEM, Invitrogen) supplemented with 30% feal bovine serum (FBS, Sigma), 10^{-11} M dexamethasone (Sigma), 1% anibiotic-antimycotic solution (ABAM, Sigma) and 1% L-lutamine (Invitrogen) [40]. The mixture was filtered through a 70 µm cell strainer, washed twice in phosphate buffered saline (PBS), and centrifuged at 400 g for 5 min at room temperature (RT). Finally, the cell pellet was resuspended in culture medium, seeded in a 25 cm^2 culture flask and cultured at 38.5 °C in a humidified atmosphere containing 5% CO_2.After 24 h, non-adherent cells were removed by replacing the culture medium. Culture medium was replaced 2–3 times a week and cells were passaged at 70–90% confuency using 2.5 mg/mL trypsin-0.2 mg/mL ethylenediaminetetraacetic acid (EDTA, Sigma). Cell viability and concentration were determined by trypan blue exclusion using a Neubauer hemocytometer. From passage (P)1 onward, cells were seeded at a density of 2,500-5,000 cells/cm^2 and sub-cultured at 70–90% confuency in expansion medium (consisting of LG-DMEM, 20% FBS,1% L-gltamine, and 1% ABAM. An overview of the experimental design is provided in Fig. 1.

Proliferation potential of bovine AT-MSCs

To evaluate proliferation, bovine AT-MSCs were continuously expanded by seeding 2,500-5,000 cells/cm^2 on 0.1% gelatin-coated tissue culture plastic and passaged upon reaching 70–90% confluency [41]. In each passage, the number of PD and PDT were recorded [13]. Cells were sub-cultured until they reached either a PDT of ≥ 3 days or 40 PD, whichever was reached first.

$$\mathrm{PD} = \frac{\ln\left(\frac{\text{number of cells harvested}}{\text{number of cells seeded}}\right)}{\ln 2}$$

$$\mathrm{PDT,\ in\ days} = \frac{\text{Time between seeding and harvesting (days)}}{\mathrm{PD}}$$

Senescence of bovine AT-MSCs

Senescence was evaluated every 3 passages starting at P3 and for as long as cells could be sub-cultured before reaching a PDT of ≥ 3 days or a maximum of 40 PD. A qualitative assessment of senescence was performed with a Senescence β-Galactosidase Staining Kit (Cell Signaling Technology). The assay is based on the reaction of β-galactosidase with a substrate, 5-bromo4-chloro-3-indolyl P3-D-galactoside, producing a blue product at pH = 6, abundance of which correlates with replicative age of the culture [42]. Briefly, 40,000 cells were seeded in a 12-well plate, containing a gelatin coated glass coverslip. After 24–48 h, the cells were fixed and stained according to the manufacturer's instructions. The cells were counterstained with a Nuclear Fast Red solution (Carl Roth) for 5 min, washed with distilled water, and dehydrated using increasing alcohol series (50- 70%- 95%- 2 × 100%. The samples were rinsed with xylene and finally mounted with SubX mounting medium (Leica) on a glass slide. The images were acquired using a brightfield microscope at a magnification of 200x (Nanozoomer, Hamamatsu).

Quantitative assessment of senescence was performed with the CellEvent™ Senescence Green Flow Cytometry Assay Kit (Invitrogen), according to the manufacturer's instructions. The assay's principle is similar to the qualitative assay (staining), with the difference that here the

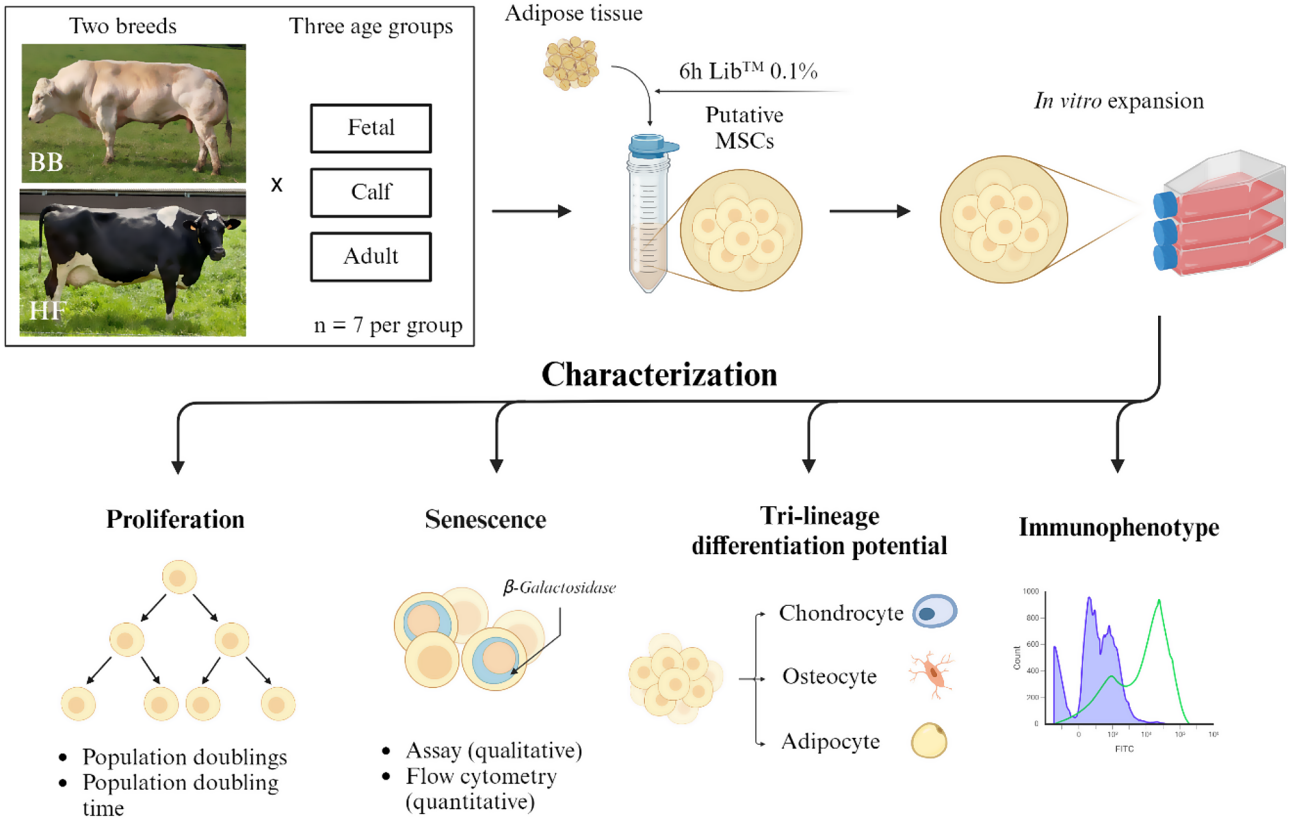

Fig. 1 Overview of the experimental design. For each AT sample ($n = 7$), bovine MSCs were isolated from HF or BB fetuses, calves, or adult cows using 6 h Liberase™ 0.1% enzymatic digestion. Subsequently, bovine MSCs were further characterized, i.e. proliferation potential (to a population doubling time (PDT) ≥ 3 days or a maximum of 40 population doublings (PD), whichever was reached first), senescence (assessed at P3, P6, P9, P12 and P15, if reached), tri-lineage differentiation ability (assessed at P4 to evaluate adipo-, osteo-, and chondrogenic differentiation potential) and immunophenotype (assessed at P4 by flow cytometry). Figure created with BioRender. HF: Holstein Friesian; BB: Belgian Blue; Lib™: Liberase™; MSCs: mesenchymal stromal cells

reaction product emits green fluorescence and is evaluated using flow cytometry. Briefly, 500,000 cells were fixed with 2% formaldehyde, washed in 1% bovine serum albumin and then incubated in airtight tubes for 1 h at 37 °C in the dark in CellEvent™ Senescence Green Probe, diluted 1:3,000 in CellEvent™ Senescence Buffer. Cells incubated in buffer without the Green Probe were used as a control. The samples were measured using a FACSVerse™ flow cytometer (BD Biosciences). After gating out cell debris, aggregates and doublets, the geomean green fluorescence intensity of the remaining cell population was measured, the control was subtracted, and the arbitrary values were reported.

Multi-color flow cytometric analysis of MSC markers

Undifferentiated bovine AT-MSCs from P4 were immunophenotyped by multi-color flow cytometry, as previously described [12]. For panel 1, approximately 500,000 cells were incubated for 30 min at 4 °C in the dark with a CD73-specific antibody (Bioss Antibodies, polyclonal, 1:50). After blocking and washing, cells were incubated with an allophycocyanin-cyanin 7 secondary antibody (APC-Cy7, AAT Bioquest, 1:100) for 20 min at 4 °C in the dark. The cells were washed and subsequently blocked for 15 min using 10%rabbit serum (Sigma). Next, the cells were incubated with a biotinylated CD90-specific antibody (Bioss Antibodies, polyclonal, 1:50). After washing, cells were incubated with a secondary Streptavidin peridinin-chlorophyll-cyanin 5.5 (PerCP-Cy5.5, Invitrogen, 1:100) together with the directly labeled primary monoclonal antibodies CD29-APC (BioLegend, TS2/16, 1:50), CD45-FITC (fluorescein isothiocyanate, BioRad, CC1, 1:10) and MHC II-(R)PE (R-phycoerythrin, BioRad, CC158, 1:20). After washing, the cell pellets were finally resuspended in 100 µL PBS with 1 µM Sytox Blue (Invitrogen). For panel 2, approximately 500,000 cells were stained with a fixable live/dead violet stain (Invitrogen). After one washing step, cells were incubated with a CD34-specific antibody (Bioss Antibodies, polyclonal, 1:100). After blocking and washing, cells were incubated with a secondary phycoerythrin-cyanine 5 antibody (PE-Cy5, ThermoFisher, 1:100) together with the directly labeled primary monoclonal antibodies CD14-(R)PE (BioRad, CC-G33, 1:100) and CD44-FITC (BioRad, IL-A118, 1:10). Subsequently, cells were fixed and permeabilized using BD Cytofix/Cytoperm™ (BD Biosciences)

for 20 min at 4 °C. After blocking and washing with 1x Perm/Wash washing buffer (BD Biosciences), cells were incubated with a CD79α-Alexa Fluor (AF)700 antibody (BioRad, HM57, 1:50) for 20 min at 4 °C in the dark. After a washing step, the cell pellets were finally resuspended in 100 µL PBS. For both panels, at least 10,000 viable single cells were analyzed using a CytoFLEX flow cytometer (Beckman Coulter), and the data were subsequently analyzed in the CytExpert software. All data were corrected for autofluorescence, compensated, and compared to specific fluorescence minus one controls.

Tri-lineage differentiation potential of bovine AT-MSCs

Undifferentiated bovine AT-MSCs of P4 were differentiated towards the adipogenic, chondrogenic, and osteogenic lineage, respectively, as routinely performed in our lab [12, 39, 43]. Non-induced bovine AT-MSCs cultured in expansion medium were used as negative controls. Briefly, adipogenic differentiation was performed in 24-well culture dishes with 21,000 cells/cm² cultured in expansion medium until 90–100% confluency. Hereafter, adipogenic differentiation was induced by subsequent cycles of 72 h culturing in adipogenic induction medium (LG-DMEM supplemented with 1 µM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine (Sigma), 10 µg/mL rh-insuline (Sigma), 0.2 mM indomethacin (Sigma), 15% rabbi serum (Sigma), 50 µg/mL gentamycin (Gibco) and 1% ABAM)followed by 24 h of culturing in the adipogenic maintenance medium (identical to the adipogenic induction medium except for the omission of dexamethasone, indomethacin, and 3-isobutyl-1-methylxanthine). After 14 days, adipogenic differentiation was assessed using Oil Red O histological staining with a Mayer's modified hematoxylin counterstaining (Abcam).

Chondrogenic differentiation was induced using a micromass culture system in which 250,000 cells were cultured in 0.5 mL of chondrogenic medium (basal differentiation medium (Lonza), supplemented with 10 ng/mL transforming growth factor-β3 (Lonza)). Upon 21 days of culture, micromass cultures were fixed overnight with 4% formaldehyde (Carl Roth), and the pellets were further processed for paraffin sectioning. Chondrogenic differentiation was evaluated by histological Alcian blue (Sigma) staining, with 0.1% Nuclear Fast Red (Sigma) counterstaining.

Osteogenic differentiation was performed in 24-well culture plates with 10,000 cells/cm² cultured in expansion medium until 90–100% confluency. Subsequently, osteogenic differentiation was induced with osteogenic medium (LG-DMEM supplemented with 10% FBS, 0.05 mM L-ascorbic acid-2-phosphate (Sigma), 100 nM dexamethasone, 10 mM β-glycerophosphate (Sigma), 50 µg/mL gentamycin and 1% ABAM), which was changed every 3–4 days. Osteogenic differentiation was evaluated after 21 days of culture using Alizarin Red S histological staining (Sigma) according to the manufacturer's instructions.

Images of adipogenic and osteogenic differentiation were captured using an inverted microscope (DMi1, Leica Biosystems, Nussloch, Germany) at a magnification of 200x and 100x, respectively. Chondrogenic differentiation images were captured at 200x using a bright field microscope (DM LB2, Leica Biosystems, Nussloch, Germany). The tri-lineage differentiation potential of bovine AT-MSCs was quantified using a differentiation ratio, obtained by dividing the area % of the differentiation signal by the area % of the nuclear signal, as previously described [43].

Data analysis

Most of the data contains at least one non-normally distributed group, which was confirmed after conducting Shapiro tests and examining Q-Q plots. Only for the parameters (i) PDT and (ii) days to reach a certain number of PD, presented in Table 1, the normal distribution was confirmed and the data are reported as mean ± standard deviation. The rest of the data is presented as median [quantile 25% - quantile 75%]. Survival analysis was performed to compare MSC proliferation capacities using the Kaplan-Meier estimator, where 1 = PD event where the culture was stopped due to PDT exceeding 3 days, and 0 = the culture reached at least 40 PD.

Group comparisons in non-normally distributed data were performed using the non-parametric Kruskal-Wallis test with Dunn's post-hoc test and Bonferroni correction for multiple comparisons. Correlations between different parameters were identified using the Spearman correlation method. All data were analyzed and visualized using RStudio version 2023.06.2 (packages: survival, ggplot2, dplyr, dunn.test).

The work has been reported in line with the ARRIVE guidelines 2.0.

Results

Effect of age and breed on bovine AT-MSC proliferation potential

To evaluate the effect of age and breed on MSC proliferation potential, bovine AT-MSCs were continuously passaged, while recording their PD number and PDT with each passage. These data were analyzed using survival analysis, in which an event was recorded every time a culture was stopped due to high PDT. The Kaplan-Meier survival curves (Fig. 2A) show the estimated "culture survival" probability over time in each group ($n = 7$). The median survival of fetal MSCs was 22 PD for the BB breed and 33 PD for the HF breed ($p > 0.05$). The survival of the calf MSCs was significantly different between the breeds: 12 PD for BB and 40 PD for HF ($p = 0.028$).

Table 1 Proliferation dynamics of bovine AT-MSC per group. Data is reported as the mean ± standard deviation. PDT rows exclude values that exceed 3 days. Days to reach a number of PD were interpolated from proliferation data of each biological replicate via a linear function. *N* = 7 unless otherwise stated

	Belgian Blue			Holstein Friesian		
	Fetal	**Calf**	**Adult**	**Fetal**	**Calf**	**Adult**
PDT, days						
P3	0.81 ± 0.12	1.22 ± 0.32	1.19 ± 0.56	1.21 ± 0.22	0.86 ± 0.2	1.40 ± 0.56
P6	1.32 ± 0.32	1.66 ± 0.65 (*n* = 5)	1.72 ± 1.12	0.91 ± 0.12	1.09 ± 0.42	1.18 ± 0.12
P9	2.01 ± 1.03 (*n* = 3)	1.81 ± 0.65 (*n* = 2)	1.48 ± 0.39 (*n* = 4)	1.77 ± 0.58	1.33 ± 0.46 (*n* = 6)	1.74 ± 0.69 (*n* = 4)
P12	1.38 (*n* = 1)	1.45 (*n* = 1)	2.41 ± 0.83 (*n* = 3)	1.93 ± 0.69 (*n* = 4)	1.84 ± 0.17 (*n* = 6)	1.79 ± 2.02 (*n* = 3)
P15	NA	1.27 (*n* = 1)	1.18 ± 0.15 (*n* = 2)	1.57 ± 0.2 (*n* = 2)	2.33 ± 1.02 (*n* = 5)	1.14 (*n* = 1)
Days to reach						
10 PD	15.3 ± 4.81	23.34 ± 5.44 (*n* = 5)	16.76 ± 5.17	10.42 ± 0.81	15.48 ± 1.56	16.98 ± 3.56
20 PD	26.48 ± 8.81 (*n* = 5)	31.44 ± 10.06 (*n* = 2)	23.46 ± 3.87 (*n* = 5)	20.06 ± 2.66	25.57 ± 4.44 (*n* = 6)	35.64 ± 10.0(*n* = 6)
30 PD	28.78 (*n* = 1)	35.14 (*n* = 1)	38.78 ± 11.73 (*n* = 3)	33.54 ± 4.49 (*n* = 4)	37.37 ± 5.72 (*n* = 6)	42.69 ± 7.02 (*n* = 3)
40 PD	41.57 (*n* = 1)	50.72 (*n* = 1)	51.56 ± 4.8 (*n* = 2)	46.81 ± 4.34 (*n* = 3)	56.79 ± 5.29 (*n* = 6)	56.9 ± 9.35 (*n* = 2)
Maximum number of PD (mean ± SD)	26.06 ± 8.02	20.92 ± 13.1	30.76 ± 8.82	33.25 ± 7.17	38.00 ± 6.16	29.73 ± 9.5
Maximum number of PD (median [Q1– Q3])	21.97 [19.7-26.78]	11.81 [9.8-19.41]	27.63 [20.54–35.28]	32.98 [26.56-40]	40.00 [39.34-40]	20.81 [20.26–37.3]
Number of donors reaching 30 doublings	1/7	1/7	3/7	4/7	6/7	3/7

PDT: population doubling time; PD: population doubling; P: passage; SD: standard deviation

The survival of the adult MSCs was similar between the breeds: 28 PD and 21 PD for BB and HF, respectively ($p > 0.05$).

Generally, isolated primary cells had a PDT between 0.8 and 1.4 days in the first passages, gradually slowing their doubling rate to 1.1–2.4 days in P12-15 (Table 1). Fetal MSCs divided remarkably faster and reached 10 PD on average within 15.3 ± 4.8 and 10.4 ± 0.8 days of culture for the BB and HF breeds, respectively (Fig. 2B; Table 1). Overall, AT-MSCs from younger donor groups (fetal and calf) proliferated faster, except for calf BB-MSCs. The latter divided slower in the first 10 PD than the other groups, and only 2 out of 7 donors reached 20 PD (Table 1). Once PDT exceeded 3 days, a sharp increase in PDT was observed indicating the onset of terminal senescence (Fig. 2D).

Regarding the maximum number of PD, fetal and calf HF cells proliferated significantly more than fetal and calf BB cells, respectively ($p < 0.001$). In adult cells, the breed effect was not significant, while inter-donor variability was higher. Within the HF breed, calf cells reached a higher maximum number of PD than both fetal cells (by 3.8 PD, $p < 0.001$) and adult cells (by 6.4 PD, $p < 0.001$). Within the BB breed, adult cells reached the highest number of PD compared to the fetal cells (by 2.6 PD, $p = 0.01$) and calf (by 5.22 PD, $p < 0.001$) (Fig. 2C).

Senescence in expanding MSCs as determined by β-galactosidase activity

To evaluate the number of senescent cells among AT-MSCs and monitor their presence in long-term cultures, we regularly performed qualitative and quantitative analyses. Accumulation of β-galactosidase and larger cell size were observed more frequently in cells of higher passages (> P12) (Fig. 3A). For quantitative analysis of senescence, a geomean difference of the green fluorescence intensity was measured between the test sample and its control (arbitrary value, AV) (Fig. 3B). Additionally, a linear mixed model was used to analyze the temporal relationship between MSC senescence and PD number. Senescence increased during proliferation (Fig. 3C). However, there were no significant differences in β-galactosidase activity between breed and age groups, and a high variability between donors was observed. Freshly isolated cells were predicted to express β-galactosidase corresponding to 117.6 AV (linear mixed model intercept = 117.6 AV, $p = 0.002$), i.e. the predicted baseline level of β-galactosidase expression in MSCs in the initial state, before any cell doubling has occurred.

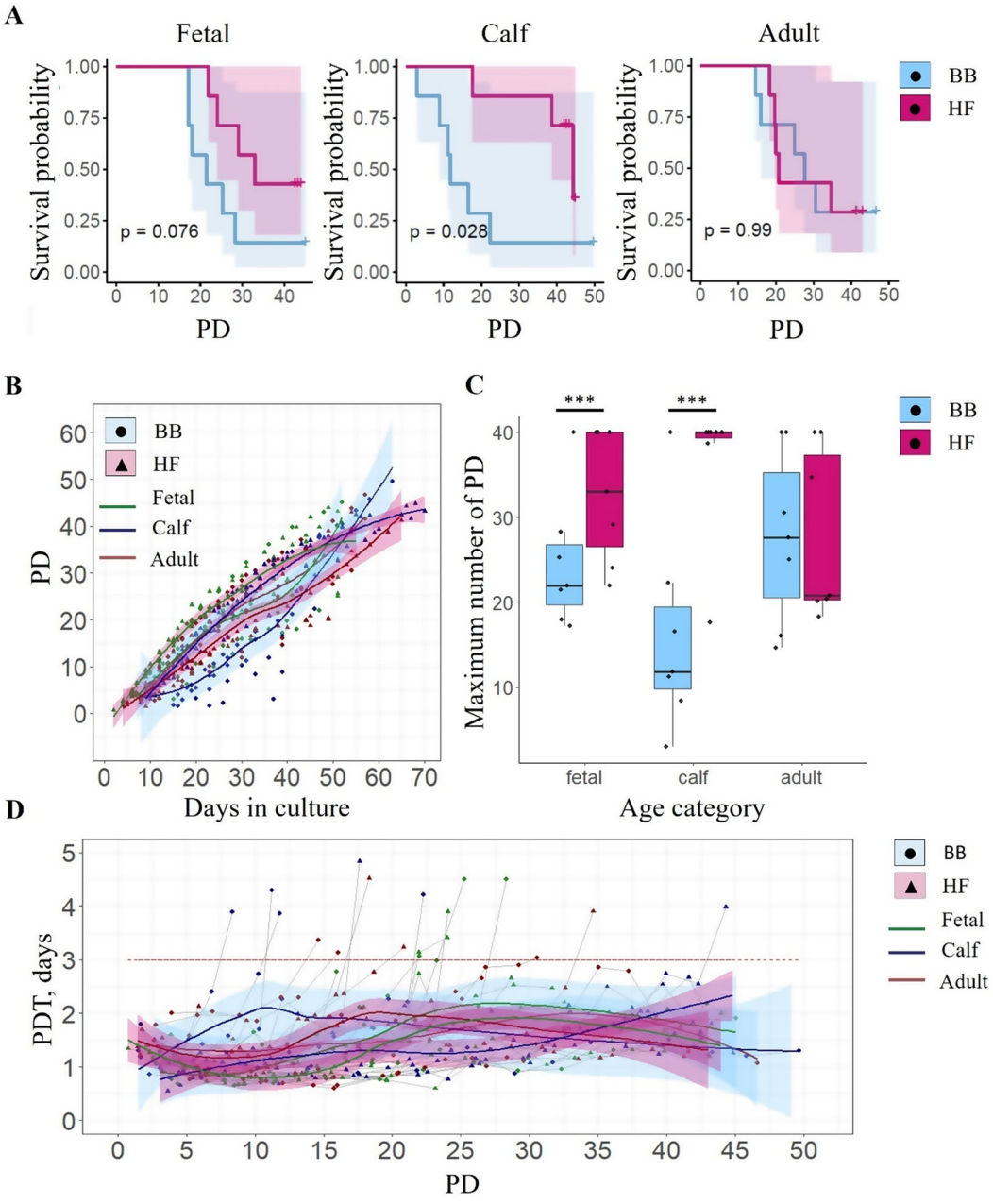

Fig. 2 Proliferation dynamics of bovine AT-MSCs isolated in two breeds and three age categories. (**A**) Kaplan-Meier survival curves representing the probability of bovine AT-MSCs achieving successive PD across all groups ($n = 7$). (**B**) PD of each group as a function of time. The curves and confidence interval are plotted using locally estimated scatter plot smoothing (LOESS) to illustrate trends and confidence intervals for proliferation dynamics. Data points represent individual donor samples for each breed and age group. (**C**) Maximum recorded number of PD before cells reached a PDT of 3 days or 40 PD. Boxplots represent medians and interquartile range. (**D**) PDT of each group plotted as a function of PD using LOESS. Individual connecting lines represent the cultures of each donor. A PDT of 3 days is highlighted as a threshold value, which, when crossed by a given culture, can be considered as its proliferation limit, as illustrated by the steep increase in PDT. PD: population doubling; PDT: population doubling time; HF: Holstein Friesian; BB: Belgian Blue

This value increased by 4.4 AV with each PD (slope = 4.4 AV, $p = 0.005$), which means that for each additional doubling of the MSC population, the expression level of β-galactosidase increased. Due to the high inter-donor variability, the model also includes a random intercept for separate donors (1|donor), this way the model's intercept can be adjusted for individual donors depending on their own baseline level of β-galactosidase expression.

Effect of age and breed on cell surface markers

Based on two multi-color panels, the percentage positivity of surface markers used to characterize bovine AT-MSCs was evaluated. As illustrated in Fig. 4A, bovine

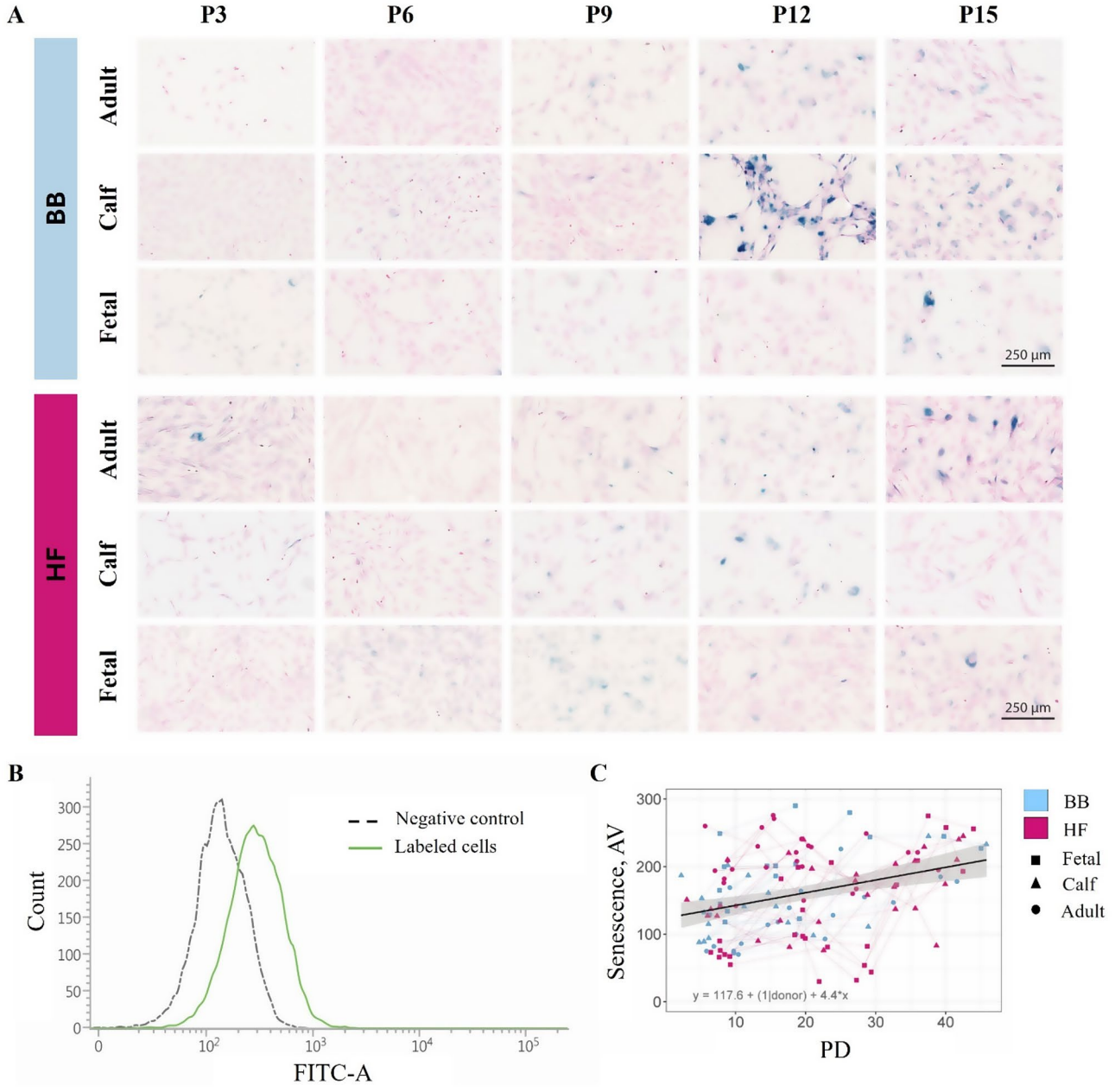

Fig. 3 β-galactosidase-associated senescence in bovine AT-MSCs. (**A**) Representative microscopic images showing qualitative analysis of senescent cells stained for β-galactosidase activity (blue) and nuclear counterstaining (pink). The images illustrate differences in senescence levels across samples, providing a visual representation of the cellular morphology associated with senescence. Scale bar = 250 µm. (**B**) Representative example of flow cytometric analysis used to quantify β-galactosidase-associated senescence. The graph depicts the distribution of green fluorescence intensity in a negative control sample compared to a test sample. Arbitrary values (AV) represent the geometric mean fluorescence intensity difference between the test sample and its matched negative control, reflecting the level of senescence in the test sample. (**C**) Quantitative analysis of senescence correlated with PD number. The plot integrates data from all groups, with senescence levels increasing with higher PD numbers. The black line represents the prediction model generated using a linear mixed-effects model to describe the relationship between senescence and PD number. Individual data points reflect senescence levels measured at specific PD, highlighting group variations. PD: population doubling; P: passage; HF: Holstein Friesian; BB: Belgian Blue

AT-MSCs from all groups showed a comparable percentage of positive cells for CD14, CD29, CD44, CD45, CD73, CD79α, CD90 and MHC-II (Fig. 4A). Regardless of donor age and breed, MSCs were strongly positive for CD29 and CD44, showed a variable presence of $CD34^{+}$ and $CD90^{+}$ cells, and were negative for CD14, CD45, CD73, CD79α, and MHC-II (Fig. 4A). As there was a clear variation between the different age and breed groups in the expression of CD34, CD44, and CD90, the effect of age and breed on these markers was further studied (Fig. 4B-D).

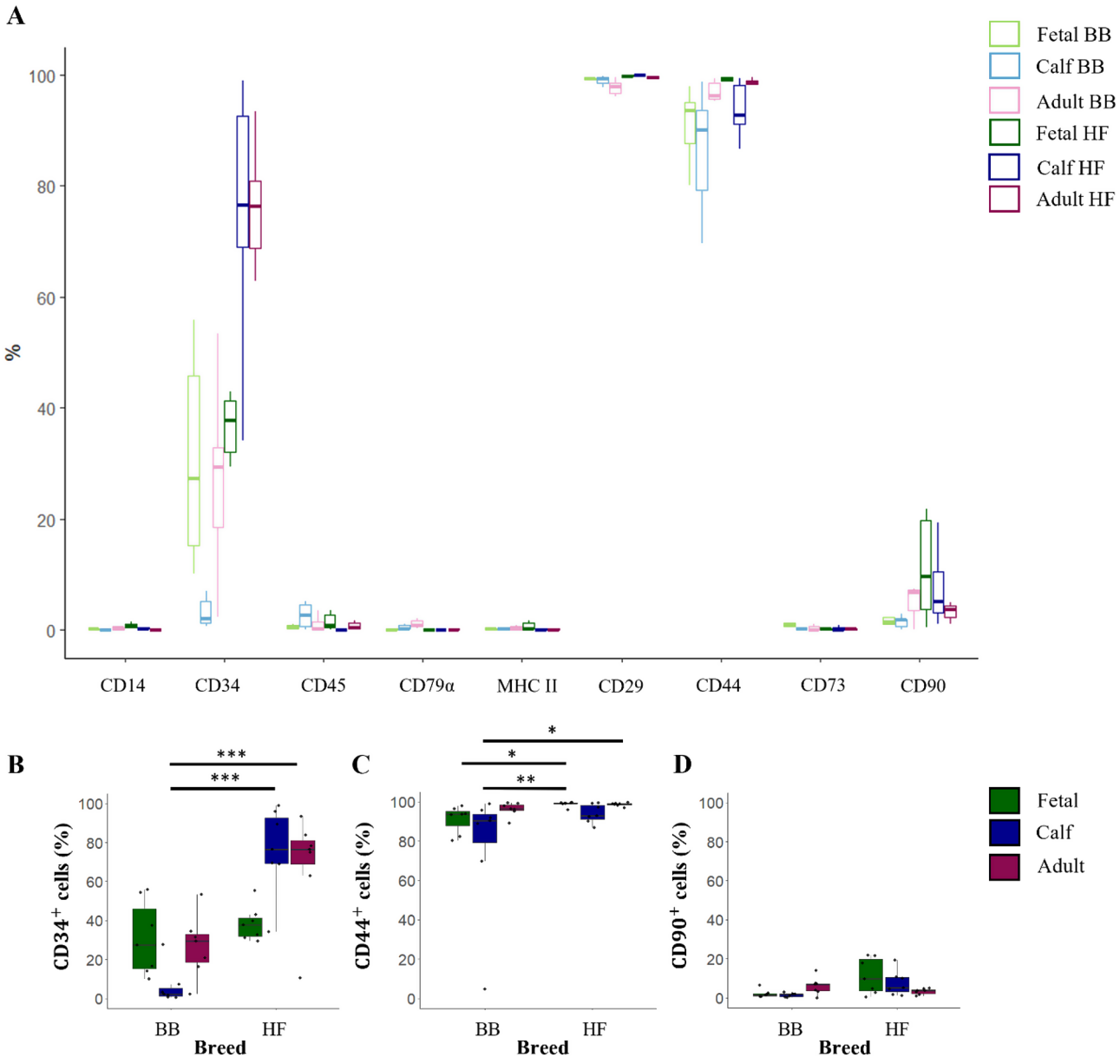

Fig. 4 Immunophenotypic profile of bovine AT-MSCs. (**A**) Overview of MSC marker expression levels with bovine MSCs from all ages and both breeds expressing high CD29 and CD44 levels, variable CD34 and CD90 levels, and low CD14, CD45, CD79α, MHCII and CD73 levels. Quantitative data for markers (**B**) CD34, (**C**) CD44, and (**D**) CD90 are presented as the median percentage of positive cells, while the bars show the interquartile range. HF: Holstein Friesian; BB: Belgian Blue

Interestingly, an effect of breed was observed for the percentage of $CD34^+$ and $CD44^+$ MSCs. Calf HF-MSCs contained significantly more $CD34^+$ cells compared to calf BB-MSCs (35.6-fold higher, $p = 0.0001$) (Fig. 4B). Fetal BB-MSCs showed a significantly higher number of $CD44^+$ cells compared to fetal HF-MSCs (1.06-fold higher, $p = 0.0256$) (Fig. 4C). Furthermore, significantly more $CD44^+$ cells were observed for fetal HF compared to calf BB (1.10-fold, $p = 0.005$) (Fig. 4C). No significant effects of breed or age were observed for CD90 expression (Fig. 4D) ($p > 0.05$).

Effect of age and breed on tri-lineage differentiation potential

To confirm their MSC identity, cells were differentiated towards adipocytes, osteocytes, and chondrocytes, respectively. Based on a qualitative and quantitative evaluation of the respective histological stainings, the tri-lineage differentiation potential was confirmed for all donors (Fig. 5). Quantitative data was obtained using an image-based analysis by dividing the area percentage of the differentiation signal by the area percentage of the nuclear signal or the cell culture area [43]. The

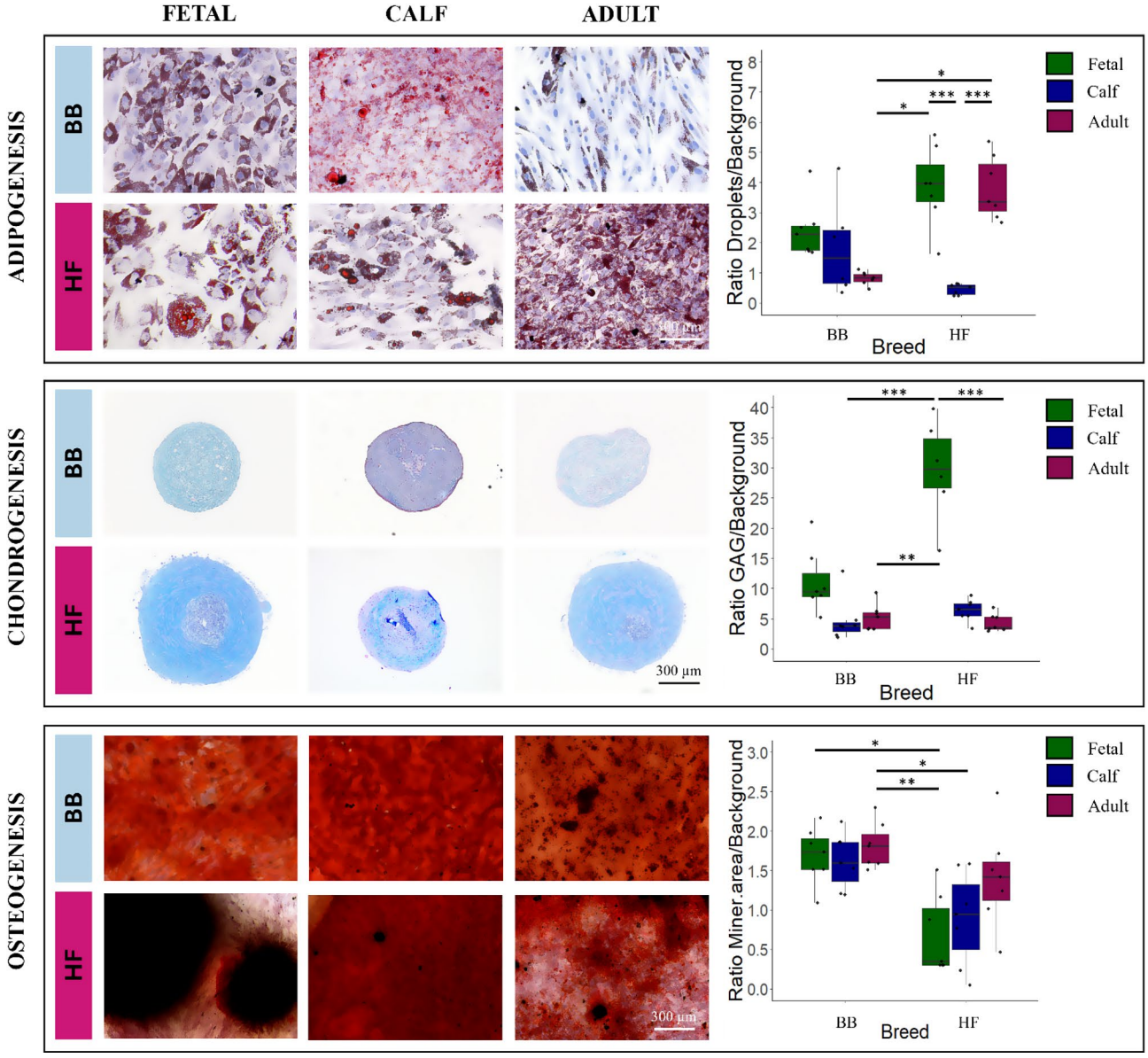

Fig. 5 Tri-lineage differentiation potential of bovine AT-MSCs. Representative images and quantitative analyses demonstrate the ability of bovine AT-MSCs to differentiate into adipogenic, chondrogenic, and osteogenic lineages, confirming their multipotency. Adipogenic differentiation is shown by the accumulation of lipid droplets stained with Oil Red O. Chondrogenic differentiation is demonstrated by Alcian Blue staining, showing deposition of glycosaminoglycans (GAG) in the extracellular matrix. Osteogenic differentiation is shown by Alizarin Red S staining, indicating calcium deposits formed during mineralization. Quantitative data for each group are presented as the median of the differentiation ratio (obtained by dividing the area % of the differentiation signal by the area % of the nuclear signal), while the bars show the interquartile range. Scale bar = 300 µm. HF: Holstein Friesian; BB: Belgian Blue; GAG: glycosaminoglycans

adipogenic differentiation ratio was significantly higher in differentiated fetal and adult HF-MSCs compared to differentiated calf HF-MSCs (7.6-fold higher, $p = 0.0006$ and 6.5-fold higher, $p = 0.0005$, respectively). Additionally, adipogenic differentiation was significantly lower in differentiated adult BB-MSCs as compared to differentiated adult (4.0-fold, $p = 0.0369$) HF-MSCs (Fig. 5). The chondrogenic differentiation ratio was significantly higher in differentiated fetal HF-MSCs compared to differentiated adult HF-MSCs (8.8-fold higher, $p = 0.0004$). A trend of decreasing glycosaminoglycan content was observed with increasing donor age for HF-MSCs (Fig. 5). There was also an effect of breed for the osteogenic differentiation potential. Calcium deposition was significantly higher in differentiated fetal BB-MSCs compared to differentiated fetal HF-MSCs (5-fold higher, $p = 0.0396$).

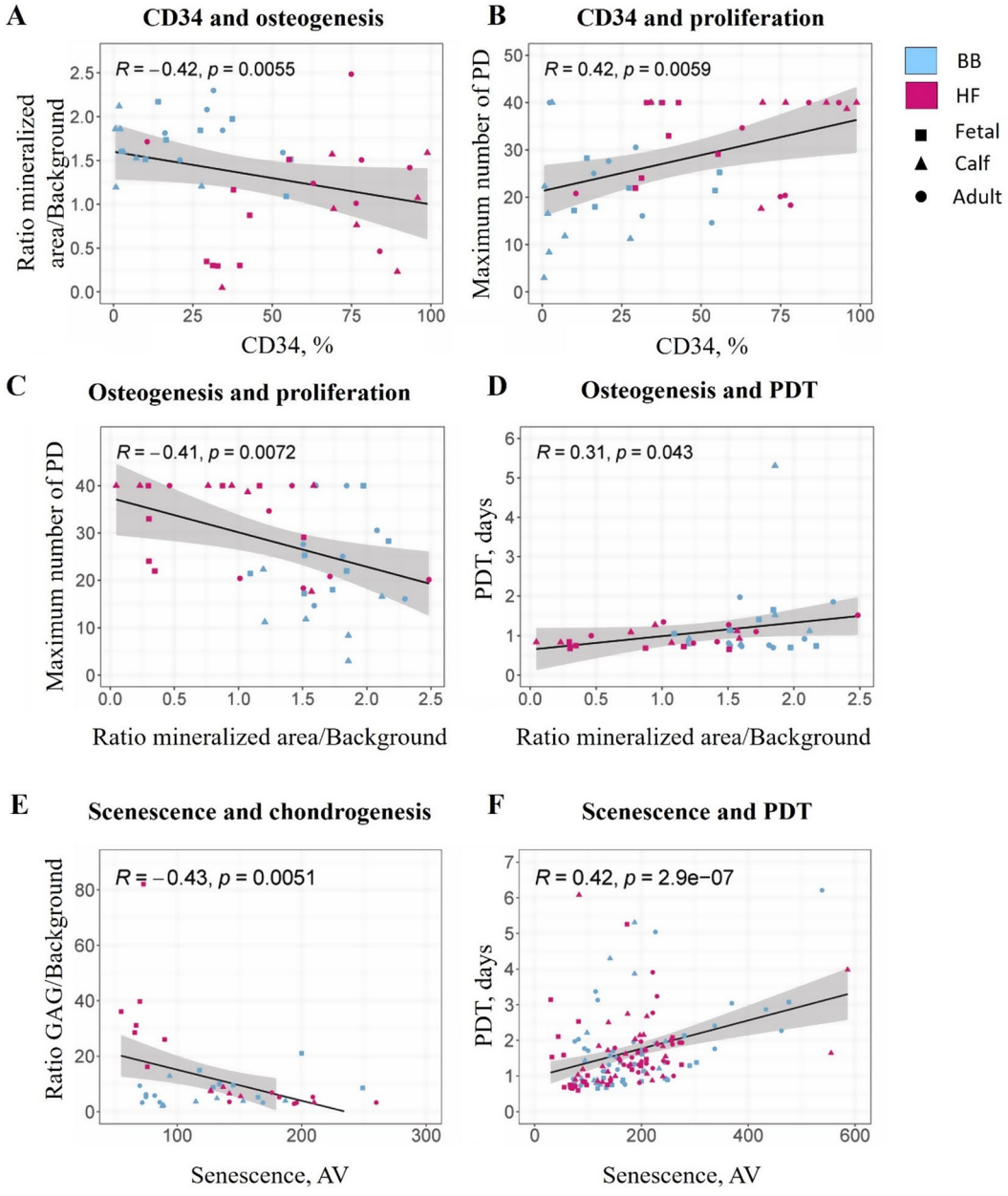

Fig. 6 Correlation analysis between different parameters of bovine AT-MSCs. The figures illustrate Spearman's rank correlation analysis, revealing significant relationships between immunophenotypic, proliferative, differentiation, and senescence parameters of bovine AT-MSCs. Correlation trends are represented by scatter plots, with each data point corresponding to individual samples across breeds (HF and BB) and age groups (fetal, calf, and adult). The fitted lines depict the direction and strength of correlations, and R values indicate Spearman's correlation coefficients. (**A**) MSCs showing a high CD34 expression, have a lower osteogenic differentiation potential (**B**) but a higher proliferation potential. (**C**) MSCs showing a higher osteogenic differentiation potential, have a lower proliferation potential (**D**) resulting in a higher PDT at P4. (**E**) Senescent MSCs show a lower chondrogenic differentiation potential at P4 (**F**) and a higher PDT. Arbitrary values (AV) of senescence represent the geometric mean difference between the green fluorescence intensity of a test sample and its control. PD: population doubling: PDT: population doubling time, AV: arbitrary value; BB: Belgian Blue; HF: Holstein Friesian

Correlations between bovine AT-MSC characteristics

Understanding the relationships between variables can provide insights into the behavior of MSC. For example, $CD34^+$ MSCs were reported to exhibit a higher proliferation potential, while $CD34^-$ MSCs showed a greater ability for adipo- and osteogenic differentiation [15]. To this end, correlation analyses were performed.

While CD34 expression is negatively correlated with osteogenic differentiation potential (Spearman's R = -0.42, p = 0.006) (Fig. 6A), it is positively correlated with the maximum number of PD (Spearman's R = 0.42, p = 0.006) (Fig. 6B). In line with these data, osteogenic differentiation potential was negatively correlated with the maximum number of PD (Spearman's R = -0.41,

$p = 0.007$) (Fig. 6C). A longer PDT of MSCs was positively correlated with osteogenic differentiation potential (Spearman's $R = 0.31$, $p = 0.043$) (Fig. 6D).

The chondrogenic differentiation potential of bovine AT-MSCs was negatively correlated with senescence (Spearman's R = -0.43, $p = 0.005$) (Fig. 6E). However, this correlation is mainly driven by the fetal HF-MSCs, showing exceptionally low senescence before the induction of chondrogenic differentiation, and demonstrating the highest accumulation of glycosaminoglycans (Fig. 6E). As expected, cells with higher senescence also showed a higher PDT (Spearman's $R = 0.42$, $p = 2.9 \times 10^{-7}$) (Fig. 6F). Other correlations tested were not significant.

Discussion

In this study, we demonstrated that donor characteristics such as age and breed clearly affect MSC proliferation rate, differentiation potential, and immunophenotype. Many regenerative applications require a significant number of cells [44, 45], which is a limitation when primary cell cultures are used, as most adult cell types have a limited lifespan [46]. Here, fetal and calf HF-MSCs showed the highest proliferation capacity compared to adult HF-MSCs and all age categories of BB-MSCs, even surpassing 30 PD. This is in line with what is generally accepted for regenerative or tissue engineering applications [44, 45, 47]. When specifically considering bovine MSC applications, these results indicate that preferably HF-MSCs should be used to produce cultured meat, a process that also requires a high number of cells [39]. In the study by Gençer et al. (2024), fetal human MSCs also showed increased proliferation when compared to adult MSCs [48]. Also studies in other species (equine, porcine, and rodents) indicated similar age-associated declines in MSC proliferation and differentiation, together with increased senescence [19, 22, 49, 50]. These parallels highlight conserved biological mechanisms underlying MSC aging across species.

After repeated passaging, MSCs can enter a state of replicative senescence, also known as the Hayflick limit [51–53], which was also observed in our study by the increased β-galactosidase activity in cells with higher doubling number. It has already been reported that the state of replicative senescence can affect the differentiation potential of MSCs [54]. Our study confirmed that there is a negative correlation between senescence and chondrogenic differentiation of MSCs although we assessed differentiation potential at P4, when senescence is not yet as pronounced. While fetal HF-MSCs indeed exhibited low senescence and the highest chondrogenic differentiation potential, adult BB-MSCs showed both low senescence and low chondrogenic differentiation potential. Therefore, low senescence might be mandatory, but not the only factor affecting chondrogenic differentiation potential of MSCs.

The observed differences in proliferation and differentiation capacities between breeds may occur due to breed-specific genetic and metabolic factors [55]. The terms secretion type and accretion type are often used in the context of cattle breeding to describe different growth patterns in relation to their production and physiology [56, 57]. HF cattle is the example par excellence of a secretion type as they are specifically reared and selected for high milk production. The focus of these breeds is on optimizing the secretion of milk components (e.g., fat, protein, lactose) rather than tissue deposition for meat. They are characterized by higher metabolic activity, which might explain the higher proliferation potential of HF when compared to BB, which is a typical beef breed. Indeed, lower basal glucose concentrations have been reported for BB calves when compared to HF calves [56, 58]. As glucose is a primary energy source for many cells, reduced glucose availability can limit energy production through glycolysis and other metabolic pathways, resulting in slower cell growth and proliferation. Hence, lower basal glucose concentrations in BB results in slower cell growth and proliferation, explaining the observed differences in proliferation potential between both breeds.

The BB cattle on the other hand is the example par excellence of the accretion type which growth is characterized by the deposition of body tissues, such as muscle and fat, rather than secretion-based productivity. Besides their lower metabolic demand compared to HF, they also have a more robust and compact body conformation. The fact that they are more focused on the deposition of body tissues than HF, might explain why the decline in chondrogenic differentiation is less pronounced in BB when compared to HF since BB might be more focused on the production of cartilage. Future studies might further explore the breed effect on bovine AT-MSC characteristics.

Regarding the immunophenotype, MSCs of different age categories and breeds showed a similar profile, being strongly positive for CD29 and CD44, and negative for CD14, CD45, CD73, CD79α, and MHC class II. Interestingly, CD34 expression varied significantly between breeds. We also showed that CD34 expression was positively correlated with the maximum number of PDand negatively correlated with osteogenic differentiation potential. Similar observations have been reported by other studies [15, 59, 60]. For instance, human AT-derived $CD34^+$ MSCs exhibited a higher proliferation potential, while $CD34^-$ MSCs showed a greater ability for adipogenic and osteogenic differentiation [15]. In line with our study, calf HF-MSCs were highly positive for CD34, and showed explicit proliferation potential, compared to $CD34^-$ calf BB-MSCs, which showed more

osteogenic differentiation potential. These observed correlations highlight the importance of understanding MSC heterogeneity as this variability reflects distinct subsets of MSCs with different differentiation and proliferation properties. The results of our study indicate that donors might be selected based on CD34 expression when considering therapeutic applications. For example, selecting MSCs with lower CD34 expression may improve therapeutic outcomes when applied in orthopedic cases, while donors with a high CD34 expression might be selected when a high number of MSCs is required, e.g. to produce cultured meat. Pre-screening donors based on CD34 expression has already been reported in literature as MSCs with low CD34 expression may exhibit strong immunomodulatory properties as well, making them suitable for autoimmune or inflammatory conditions [61].

Regarding the tri-lineage differentiation potential, adipogenic differentiation was significantly higher in fetal and adult HF-MSCs compared to calf HF-MSCs. In contrast, Stolzing et al. (2008) reported no age-related changes in the adipogenic differentiation potential of human BM-derived MSCs (young: 2.5-9 years vs. adult: 18–55 years) [62]. An increase in the adipogenic differentiation potential of fetal and adult bovine HF-MSCs may be explained by the dynamic process of adipogenesis during development. In human fetuses, the greatest increase in adiposity occurs in the third trimester of pregnancy, correlated to an exponential increase in the number of adipocytes through differentiated MSCs. During bovine tissue sampling, we also observed a higher amount of subcutaneous AT in third-trimester bovine fetuses. After birth, low AT accumulation rates are sustained until the onset of puberty, at which point the pattern of AT accumulation is determined by factors such as diet, sex, and the gut microbiome [63]. However, in BB we did not observe such an age effect on adipogenic differentiation potential. Moreover, adult BB-MSCs showed a lower adipogenic differentiation potential than adult HF-MSCs. This can be explained by the double-muscling trait in BB, as a result of loss of myostatin function, which has higher muscle-to-fat ratios [64, 65], resulting in lean meat with less fat (i.e. reduced number of adipocytes through differentiated MSCs), compared to HF. This is in line with the study of Artaza et al. (2005) which showed that loss of myostatin function directly suppressed adipogenesis in murine MSCs [66].

For HF-MSCs, we observed that glycosaminoglycan content decreased as donor age increased, supporting the hypothesis that increasing donor age is a major variable affecting chondrogenic differentiation in vitro, which has also been reported for other species [19, 62].

Regarding osteogenic differentiation potential, no effect of age could be observed, as also showed in a previous study in which adult rabbit BM-MSCs (4–5 years) were capable of osteogenic differentiation at levels similar to those of young cells (4–6 months) [67]. Interestingly, an effect of breed could be observed for osteogenic differentiation, which confirms the correlation we observed between CD34 expression and differentiation potential, as BB-MSCs showed a lower percentage of $CD34^+$ cells and a higher osteogenic differentiation capacity compared to HF-MSCs. Furthermore, as a result of loss of myostatin function, osteogenic differentiation may be increased which has also been observed for BM-MSCs from myostatin-deficient mice [68]. Additionally, the osteogenic differentiation potential (at P4) was negatively correlated with the maximum number of PD. This negative correlation is a common feature in stem cell biology, as it ensures a balance between maintaining a pool of stem cells while producing differentiated cells, for example, required for proper bone formation, to maintain homeostasis [69].

It should be mentioned that, in addition to age and breed, the sex of the donor might influence the immunophenotype and differentiation potential of MSCs although this was not taken into account in our study due to constraints in sample availability. We sampled only male calf donors and only female adult donors in both breeds, enabling a proper breed comparison within these age groups. The fetal age group, however, consisted of both sexes as we could only occasionally collect them. We realize that the comparison of the age groups in the present study may be confounded by a possible sex effect. However, based on a comparison of data from both sexes in the fetal HF-MSCs group (consisting of 3 male and 4 female donors), no statistically significant differences were observed in any of the measured parameters (data not shown). Taking the limited sample size into account, the sex effect on MSC characteristics should however be explored in future studies.

Conclusions

This study clearly shows the impact of age and breed on the immunophenotype, proliferation, and differentiation potential of bovine AT-MSC. As cells of animal origin are frequently used in veterinary regenerative studies and as relevant models for translational studies, the effects of breed and age should be taken into account. The latter is also important in order to select the most appropriate MSC donor in allogeneic MSC-based therapies, which will most likely improve treatment efficacy. Further research should focus on unraveling and circumventing MSC heterogeneity in order to standardize MSC therapies.

Abbreviations

ABAM	Antibiotic antimycotic solution
AF	Alexa Fluor

AT	Adipose tissue
AV	Arbitrary value
BM	Bone marrow
BB	Belgian Blue
CD	Cluster of differentiation
CI	Confidence interval
EDTA	Ethylenediaminetetraacetic acid
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
HF	Holstein Friesian
ISCT	International Society for Cell & Gene Therapy
LG-DMEM	Low glucose Dulbecco's Modified Eagle Medium
LOESS	Locally estimated scatter plot smoothing
MHC	Major Histocompatibility Complex
MSCs	Mesenchymal stromal cells
P	Passage
PBS	Phosphate buffered saline
PD	Population doublings
PDT	Population doubling time
PE-Cy5	Phycoerythrin-cyanine 5
PerCP-Cy5.5	Peridinin-chlorophyll protein-cyanine 5.5
RPE	R-phycoerythrin
RT	Room temperature

Supplementary Information

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Supplementary Table 1

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The authors declare that they have not used AI-generated work in this manuscript.

Author contributions

EH and MO were involved in conception and design, execution of experiments, data analysis, and manuscript writing. EDV was involved in execution of experiments, data analysis and manuscript editing. SDS, BD, LT and CDS were involved in funding acquisition, conception and design, data analysis and manuscript editing.

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Data availability

Data files presented in this study are available upon request, please contact the corresponding author Catharina De Schauwer.

Declarations

Ethical approval and consent to participate

Not applicable.

Consent for publication

All authors have approved the manuscript for submission.

Competing interests

The authors declare no conflict of interest.

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