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Original article

Vitamin D: Correlation with biochemical and body composition changes in a southern Brazilian population and induction of cytotoxicity in mesenchymal stem cells derived from human adipose tissue

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ABSTRACT

Studies have shown that metabolic disorders, serum inflammatory markers and weight gain (obesity) are correlated with vitamin D deficiency. Therefore, the present study correlated the serum calcidiol (s25 (OH)D_3) levels in a sample of individuals from southern Brazil with variables related to metabolic disorders, obesity and lifestyle habits and assessed the cytotoxic effect of calcitriol on adipose tissue-derived mesenchymal stem cells (ADSCs). The results showed a 79.23% prevalence of hypovitaminosis D in the study population and a correlation ($p < 0.05$) between a low serum vitamin D concentration and an elevated low-density lipoprotein cholesterol (LDL-c) level. Univariate linear regression analysis using 25 (OH)D_3 as a regressor showed a negative association ($p < 0.05$) with an indoor work environment ($\beta = -2.305$), increased body fat ($\beta = -0.095$), age ($\beta = -0.065$) and high-density lipoprotein cholesterol (HDL-c; $\beta = -0.109$). An *in vitro* 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay performed with ADSCs using five calcitriol concentrations (15.625, 31.25, 62.5, 125 and 250 nM) indicated cytotoxic potential ($p < 0.05$) at the 62.5 nM concentration at 48 and 72 h and at the 125 and 250 nM concentrations at 24, 48 and 72 h. The results reported herein corroborate one another and suggest a key association between vitamin D deficiency and the development of obesity because ADSCs are involved in adipose tissue hyperplasia and differentiate into adipocytes that can sequester the bioavailable vitamin D necessary for homeostasis.

1. Introduction

Vitamin D has become a research study focus primarily because it modulates functions related to bone health [1,2], tumour development [3–5] and metabolic disorders [6–9].

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One of the various forms of vitamin D is vitamin D_3 (cholecalciferol), which can be produced through sun exposure (endogenous production) and ingested/supplemented. Therefore, vitamin D_3 may be the most studied vitamin in medicine because it is widely used as a supplement in hypovitaminosis D cases. Through a regulated process in the body, circulating vitamin D_3 is converted to the bioactive vitamin D hormone, which is best known as calcitriol ($1,25(OH)_2D_3$). For this purpose, vitamin D_3 undergoes a hydroxylation process that primarily occurs in the liver; this process is mediated by the 25-hydroxylase enzyme, which causes the conversion to calcidiol ($25(OH)D_3$). Subsequently, the second hydroxylation mostly occurs in the kidneys and is, in turn, mediated by the 1α-hydroxylase enzyme, which converts 25 $(OH)D_3$ into $1,25(OH)_2D_3$ [10–13]. However, optimal serum levels are not reached, even with the ingestion of vitamin D_3-rich foods [14]; thus, supplementation and sun exposure are needed, preferentially during the morning period [15–17].

Vitamin D deficiency, as assessed using the serum $25(OH)D_3$ level, has been reported in children, adults and elderly people from all continents [18–20]. Because deficiency occurs worldwide [21,22], further population studies are needed even in countries thought to have a high annual solar incidence [23–26]. In Brazil, vitamin D deficiency is most worrying because this condition affects different groups of study subjects, including young healthy individuals and young people with metabolic disorders (e.g., patients with metabolic syndrome) [27–30].

Systematic reviews have shown that metabolic disorders, including cardiovascular disease (with an increasing incidence), high serum lipid concentrations, serum inflammatory markers, changes in glucose metabolism and weight gain, are correlated with vitamin D deficiency [9,31–33]. These parameters are related to obesity progression and being overweight in humans. Adipose tissue expansion is mediated by hypertrophy of mature adipocytes or multipotent stem cell hyperplasia during adipogenic differentiation with a subsequent triglyceride accumulation in adipose tissue [34,35]. Recently, stem cells adjacent to adipose tissue have been shown to play key roles in the adipose tissue mechanisms underlying chronic inflammation; therefore, these cells may be a key factor in an adjunct treatment for obesity prevention [36]. Thus, studies on the genetic cascade of the inhibition of the proliferation and/or death of adipose tissue cells have provided new possibilities for the prevention of this disease [37–40].

Vitamin D has been reported to be a mesenchymal stem cell proliferation inhibitor and an inducer of apoptosis in various cell types, including adipocytes [37,38,41–43]. Therefore, the use of vitamin D, especially in its hormone form, may be a good strategy to prevent obesity.

Considering the above findings, the present study correlated the serum calcidiol ($s25(OH)D_3$) levels in a sample of individuals from southern Brazil with variables related to metabolic disorders, obesity and lifestyle habits and assessed the cytotoxic effect of 1,25 $(OH)_2D_3$, which is commonly found in one type of medication, on adipose tissue-derived mesenchymal stem cells (ADSCs).

2. Patients and methods

2.1. Population study

2.1.1. Inclusion criteria

Adult volunteers of both genders aged from 18 to 55 years who read and signed the informed consent form and lived for at least three months in the city of Londrina, Paraná state, Brazil (Latitude: 23°18′37″ South), were included in the research study. Only self-declared Caucasian individuals participated in the research study to follow the standards of similar studies [44,45] and to avoid effects from skin vitamin D production due to excess pigmentation. The study was submitted to the Ethics Committee on Research Involving Human Subjects of the State University of Londrina (Universidade Estadual de Londrina–UEL) and approved under opinion number 116/2011.

2.1.2. Exclusion criteria

Patients with cardiovascular, gastrointestinal or kidney diseases, thyroid disorders, diabetes, haemophilia, anaemia or cancer, individuals who did not reside in the municipality of Londrina for at least three months and those who were not self-reported Caucasians were excluded from the study. Individuals who reported the chronic use of medicines to treat diabetes mellitus or dyslipidaemia or who used multivitamins in the last six months were also excluded from the study.

2.1.3. Data on the initial and final sampling

The present study was announced in the Oswaldo Cruz Laboratory (Londrina – PR) to patients waiting for the collection of their routine exams. At that time, the researcher responsible for this work invited patients to include the evaluated exams at no additional cost and explained the importance of their contribution. A total of 370 patients demonstrated an interest in participating and authorised blood collection for vitamin D and other biochemical variable dosing.

2.1.4. General data and sample collection procedures

Biological material and data on anthropometric parameters were collected over a six-month period. The collections were performed two weeks after the beginning of spring and two weeks after the end of summer in 2013.

The experiment was performed with a double-blind design. The following materials were provided to each volunteer: a standard care questionnaire, wherein personal information (name, age, gender, address, ethnicity and use of medicines) was recorded, and the shortened and modified version of the International Physical Activity Questionnaire (IPAQ; [46]). The data recorded on the latter questionnaire were based on the objectives of the study and included daily sun exposure determined by workplace specifications, sunscreen use habits and whether vigorous/moderate physical activity was performed. Additionally, the volunteer was subjected to a rapid blood glucose test (Accu-Check Active®, Roche Diagnostics, Switzerland). If the result indicated values higher than 126 mg/dL, the subject was automatically excluded from the study and referred for medical evaluation. After this initial evaluation, the subjects were referred for biological material collection and anthropometric evaluation. The aforementioned procedures were performed in the morning from 08:00 to 10:00 AM to minimise externalities that could cause variations between the tested parameters.

2.1.5. Determination of biochemical variables

The $s25(OH)D_3$ values were evaluated using an automated chemiluminescence analyser (LIAISON®, DiaSorin Diagnostics, Italy). Free fatty acids, high-density lipoprotein cholesterol (HDL-c), triglycerides and glucose were assessed using the automated enzymatic-calorimetric method (ADVIA® 1650, Siemens, Germany). Low-density lipoprotein cholesterol (LDL-c) was calculated using the Friedewald equation [47].

2.1.6. Anthropometric evaluation

For the anthropometric examination, the waist circumference was measured using an inelastic tape measure (Incoterm®, Brazil). Bioelectric impedance analysis was performed using a MALTRON® BF-906 Body Composition Analyser (Maltron International, UK) to assess the percentage of body fat and the body mass index (BMI).

2.2. In vitro *study*

2.2.1. Individuals

Individuals living in Campo Grande, Mato Grosso do Sul state, Brazil (latitude: 20°26′34″ South), who were referred for liposuction and scheduled for surgery were contacted and informed about the research study. Three adult female volunteers aged from 20 to 40 years who read and signed the informed consent form were selected. The study was submitted to the Ethics Committee on Research Involving Human Subjects of the Federal University of Mato Grosso do Sul (Universidade Federal de Mato Grosso do Sul–UFMS) and approved under opinion number 867.377.

2.2.2. Procedures for liposuction, lipoaspirate processing and mesenchymal stem cell culture

After the anaesthetic procedure, asepsis and antisepsis, surgical incisions of approximately 1.0 cm were made into the anterolateral abdominal wall to collect the biological material. Approximately 200 mL of saline solution without a vasoconstrictor was injected into the adipose stroma for tissue detachment. Then, the detached adipose tissue was collected using a liposuction cannula. This 0.5-cm medium-calibre cannula was coupled to a 50-mL syringe for fat collection. Approximately 300 mL of biological material was collected in a sterile container with 200 mL of phosphate-buffered solution (PBS; 8 g NaCl, 0.2 g KCl, 1.15 g Na_2HPO_4, 0.2 g KH_2PO_4 and 1000 mL of deionised water). Shortly thereafter, the liposuction surgical procedure was performed according to the most appropriate technique for the patient. The crude lipoaspirate of each patient was only stored to collect the data for the present study and was later discarded in a biohazard waste container, followed by hospital waste disposal. For the processing of each of the three lipoaspirate samples, collagenase was used for sample digestion as described by Markarian, Frey, Silveira, Chem, Milani, Ely, Horn, Nardi and Camassola [48] with modifications. Culture medium was composed of Dulbecco's modified Eagle's medium with 10 mM HEPES (HDMEM; Sigma® catalogue number D5523) supplemented with 10% foetal bovine serum.

The immunophenotype was characterised by flow cytometry. Cells were incubated with antibodies against phycoerythrin or fluorescein isothiocyanate-conjugated antibodies specific for human CD44, CD90, CD29, CD34, CD11b and major histocompatibility complex (MHC) II (PharMingen BD, USA) for 30 min at 4 °C. Control samples were incubated in the absence of antibodies. The cells were analysed on an ACCURI C6 (Becton Dickinson, USA). The characterisation graphs were generated with the capture of 10,000 events using the standard software for the equipment (BD ACCURI C6 software; Becton Dickinson, USA).

2.2.3. Adipogenic differentiation

Cell samples from the three patients were subjected to the adipogenic differentiation process at the fifth passage. To induce differentiation, 1.0×10^5 cells were seeded into 6-well culture plates; after cell adhesion (24 h), the cells were cultured in adipogenesis-inducing medium according to Markarian, Frey, Silveira, Chem, Milani, Ely, Horn, Nardi and Camassola [48] with modifications. In summary, 0.714 μL of insulin (Insunorm R®, Aspen Pharma/Aspen Brazil, 0.35 mg/mL), 100 μM indomethacin (0.0447 g indomethacin, Sigma® catalogue number I7378, in 5 mL of dimethyl sulfoxide (DMSO)), 3.5 μM rosiglitazone (0.0089 g of rosiglitazone, Sigma® catalogue number R2408, in 5 mL of DMSO) and 10^{-5} M dexamethasone (Sigma® catalogue number D4902) were used for every 1 mL of HDMEM supplemented with 20% foetal bovine serum. All solutions were stored in a freezer except for indomethacin, which was stored at room temperature. The differentiation medium was prepared on the day of use.

The differentiation medium was changed every 72 h [49,50], and the differentiation process lasted 14 days [51,52].

After the differentiation medium was discarded, the cells were fixed for 60 min at room temperature with 10% formaldehyde. Then, the cells were washed with isopropanol (60%) and subsequently incubated with Oil red O (Sigma® catalogue number O0625) for 20 min at room temperature to detect intracellular lipid accumulation. The excess dye was removed by washing with distilled water.

2.2.4. Osteogenic differentiation

The cells were cultured as described above for the adipogenic induction. However, the cells were grown in their own medium for osteogenic differentiation, as described by Markarian, Frey, Silveira, Chem, Milani, Ely, Horn, Nardi and Camassola [48], with modifications.

In total, 79.9 mL of HDMEM supplemented with 10% foetal bovine serum, 100 μL of ascorbic acid solution (50 mg of ascorbic acid 2-phosphate, Sigma® catalogue number A8960, in 10 mL of HDMEM), 100 mL of β-glycerophosphate stock solution (630 mg of β-glycerophosphate, Sigma® catalogue number G9891, in 20 mL of HDMEM) and 10 mL of foetal bovine serum was used to prepare the osteogenic induction medium.

The differentiation medium was changed every 72 h for four weeks. Osteogenic differentiation was demonstrated by the observation of calcium deposits stained with Alizarin Red (Sigma® catalogue number A5533) as described by Markarian, Frey, Silveira, Chem, Milani, Ely, Horn, Nardi and Camassola [48].

2.2.5. Chondrogenic differentiation

The cells were cultured as described for the adipogenic and osteogenic induction methods. After cell adhesion, the HDMEM supplemented with 10% foetal bovine serum was discarded, and the cells were cultured using a STEMPRO chondrogenic differentiation kit (Gibco® Life Technologies, catalogue number A1007101) according to the manufacturer's instructions for 21 days. The culture medium was changed every three days.

After the differentiation medium was discarded, the cells were fixed for 30 min at room temperature with a paraformaldehyde solution (4%). Then, the cells were washed twice with PBS and stained with Alcian Blue (Neon Commercial®, Brazil, catalogue number 74240) for 5 min at room temperature; excess dye was removed by washing with distilled water [53]. Differentiation was confirmed by the presence of a glycosaminoglycan-rich extracellular matrix on the 21st day.

2.2.6. Test substance: 1,25(OH)$_2$D$_3$

The commercial product Calcijex® (Abbott Pharmaceuticals, USA; 1,25$(OH)_2D_3$, 1 μg/mL) was used to assess cell viability (cytotoxic potential). This drug was chosen because it could be easily purchased from several locations worldwide, which facilitated its use in treatments.

2.2.7. MTT assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed as described by Mosmann [54] with modifications [55]. Cells from the three patients were seeded (7×10^3 cells/well, five wells per treatment) in 96-well culture plates. After a 24-h stabilisation period, the cells were separated into six groups as follows: a control group incubated with HDMEM (supplemented with 10% foetal bovine serum) alone and five treatment groups incubated with five 1,25$(OH)_2D_3$ concentrations (15.625, 31.25, 62.5, 125 and 250 nM) for 24, 48 and 72 h. At the end of the treatment periods, the treatment media were removed, and the cells were incubated at 37 °C with a mixture containing MTT (Invitrogen® catalogue number M6494; 0.005 g of MTT, 5 mL of

PBS and 10 mL of HDMEM without FBS supplementation) for 4 h. Then, the MTT mixture was removed and replaced with 200 μL of dimethyl sulfoxide (DMSO) in each well to dissolve the product (formazan crystals). Readings were performed in a spectrophotometer with a 570-nm filter. Cell viability (expressed as a percentage) was calculated according to the following formula:

$$Cell\ Viability(\%) = \frac{Mean\ Absorbance\ of\ the\ treatment\ group}{Mean\ Absorbance\ of\ the\ group\ without\ treatment} \times 100$$

2.3. Statistical analysis

The subjects of the population study were classified according to their s25(OH)D_3 levels as vitamin D-sufficient (≥ 30 ng/mL) or vitamin D-insufficient/deficient (< 30 ng/mL) subjects [24,56]. Descriptive analysis data were expressed as the mean ± standard error of the mean (SEM) or n/%, and both groups were compared using the Mann-Whitney test. Each group was separately fitted to the univariate linear regression model to examine the potential of each regressor and the behaviour of both groups when s25(OH)D_3 was used as the dependent variable. Then, the full multiple regression model was analysed by variable using a stepwise procedure. For each group, only the variables that actually contributed to a good model fit were included to provide a reduced model. Scatter plots were generated, and Pearson's correlation coefficients were calculated for s25(OH)D_3 and the clinical-laboratory and categorical variables selected after the stepwise procedure. The significance level adopted for all tests was 0.05. All tests were performed in R software *2.15.0* (R, 2011).

The MTT assay results were expressed as the percentage ± SEM. The statistical analysis was performed using analysis of variance (ANOVA)/Bonferroni's correction, with different letters indicating significant differences ($p < 0.05$; GraphPad Prism 5). A non-linear regression curve analysis was performed using the results from the test with the five 1,25(OH)$_2$D$_3$ concentrations (OriginPro® 2016) to determine the half-maximal inhibitory concentration (IC50) of 1,25(OH)$_2$D$_3$.

3. Results

3.1. Population study

The subjects were categorised according to their s25(OH)D_3 levels. A total of 79.23% of the sample consisted of vitamin D-deficient subjects (s25(OH)$D_3 < 30$ ng/mL), and 20.77% of the sample consisted of vitamin D-sufficient subjects (s25(OH)$D_3 \geq 30$ ng/mL; Table 1). The mean vitamin D concentration was 21.06 ± 0.34 ng/mL for subjects with a s25(OH)D_3 concentration <30 ng/mL and 35.77 ± 0.63 ng/mL for subjects with a s25(OH)D_3 concentration ≥ 30 ng/mL. These serum concentrations were significantly different ($p < 0.001$) and were similar to the plasma LDL-c levels, which were 113.48 ± 1.96 mg/dL in the s25(OH)$D_3 < 30$ ng/mL group and 98.41 ± 3.48 mg/dL in the s25(OH)$D_3 \geq 30$ ng/mL group ($p < 0.001$). A high coefficient of variation was observed for these and all other tested variables, indicating sample heterogeneity (Table 1).

A univariate regression analysis was performed for all variables tested according to the categorisation model. Table 2 outlines the parameter estimates, the confidence intervals of these estimates and the *p*-value of Student's *t*-test for the slope of the linear regression model for the s25(OH)$D_3 < 30$ ng/mL subjects. For this group, the following variables were significant: sun exposure (indoor work environment) ($p = 0.001$), age ($p = 0.032$), body fat percentage ($p = 0.013$) and HDL-c plasma level ($p < 0.001$). Similarly, a univariate linear regression analysis was performed for the s25(OH)$D_3 \geq 30$ ng/mL subjects (Table 3). However, no variables were significant in this regression model.

Subsequently, the variables best fitted to each categorisation model were selected. For this purpose, variables were selected from the full model using a stepwise procedure to choose the variables that best predicted each model. After the selection, a multivariate regression analysis was performed for both groups. The variables ($p < 0.05$) age, BMI, HDL-c and sun exposure (indoor work environment) were selected for the s25(OH)$D_3 < 30$ ng/mL model (Table 4). However, no variable had significant predictability ($p < 0.05$) for the s25(OH)$D_3 \geq 30$ ng/mL model (data not shown).

Table 1
Descriptive data (biochemical and anthropometric parameters) and categorization of the 337 individuals living in the city of Londrina, PR – Brazil, according to their serum calcidiol (25(OH)D_3) levels.

	s25(OH)D3 < 30 ng/mL					s25(OH)D3 ≥ 30 ng/mL					
	n = 267					n = 70					p^a
			Range	95% CI	CV (%)			Range	95% CI	CV (%)	
Gender (female, n/%)	189	70.80				52	74.30				0.565
Physical activity (not performed, n/%)	234	87.60				63	90.00				0.588
Sun exposure (indoor work environment, n/%)	169	63.30				38	54.30				0.169
Sunscreen use (yes, n/%)	78	29.21				23	32.86				0.555
Age (years, mean/SE)	33.34	0.69	17.00–55.00	31.98–34.70	33.80	32.37	1.13	19.00–55.00	30.12–34.64	29.13	0.929
Waist circumference (cm, mean/SE)	83.94	0.75	61.00–116.00	82.46–85.41	14.59	82.17	1.44	63.00–115.00	79.30–85.04	14.64	0.275
Body fat (%, mean/SE)	31.59	0.55	12.00–54.20	30.52–32.67	28.27	30.55	0.98	14.00–48.40	28.60–32.49	26.74	0.379
BMI (kg/m^2, mean/SE)	25.33	0.28	17.10–41.00	24.77–25.88	18.16	24.82	0.52	17.40–37.00	23.79–25.85	17.49	0.442
HDL-c (mg/dL, mean/SE)	54.63	0.72	30.00–97.00	53.22–56.05	21.47	54.4	1.34	36.00–89.00	51.74–57.06	20.53	0.903
LDL-c (mg/dL, mean/SE)	113.48	1.96	39.60–220.80	109.62–117.35	28.28	98.41	3.48	36.60–167.20	91.47–105.35	29.59	<0.001
Triglycerides (mg/dL, mean/SE)	102.72	3.96	28.00–445.00	94.91–110.52	63.05	98.73	8.99	26.00–449.00	80.79–116.67	76.20	0.326
Free fatty acids (nmol/mL, mean/SE)	0.55	0.01	0.21–1.28	0.53–0.57	32.73	0.56	0.03	0.25–1.33	0.51–0.61	37.50	0.692
Glucose (mg/dL, median/SE)	90.09	0.50	57.00–115.00	89.10–91.07	9.09	88.51	1.06	60.00–109.00	86.39–90.64	10.07	0.240
25(OH)D_3 (calcidiol; ng/mL, mean/SE)	21.06	0.34	4.09–29.90	20.39–21.73	26.54	35.77	0.63	30.00–52.70	34.52–37.03	14.73	<0.001

Comparative table of biochemical and anthropometric parameters categorized by the serum calcidiol levels.
[a] Statistical analysis: Mann–Whitney ($p < 0.05$). 95% confidence interval (95% CI).

Table 2
Univariate linear regression model for the subjects with an s25(OH)D_3 concentration <\30 ng/mL (subjects with calcidiol serum levels below 30 ng/mL).

	β Coefficient	95% CI		p^a
Physical activity (not performed)	−1.728	−3.768	0.312	0.097
Sun exposure (indoor work environment)	−2.305	−3.677	−0.933	0.001*
Sunscreen use (yes)	0.439	−1.044	1.923	0.560
Age (years)	−0.065	−0.125	−0.006	0.032*
Waist circumference (cm)	−0.008	−0.063	0.048	0.782
Body fat (%)	−0.095	−0.170	−0.020	0.013*
BMI (kg/m^2)	−0.097	−0.243	0.050	0.194
HDL-c (mg/dL)	−0.109	−0.165	−0.053	<0.001*
LDL-c (mg/dL)	−0.008	−0.029	0.013	0.445
Triglycerides (mg/dL)	<−0.001	−0.010	0.011	0.917
Free fatty acids (nmol/mL)	−1.917	−5.609	1.776	0.308
Glucose (mg/dL)	0.037	−0.045	0.120	0.375

[a] Statistical test: Student's *t*-test.
* Statistically significant difference ($p < 0.05$). 95% confidence interval (95% CI).

Table 3
Univariate linear regression model for subjects with an s25(OH)D_3 concentration ≥ 30 ng/mL (subjects with calcidiol serum levels above 30 ng/mL).

	β Coefficient	95% CI		p^a
Physical activity (not performed)	1.048	−3.165	5.260	0.621
Sun exposure (indoor work environment)	0.943	−1.588	3.475	0.460
Sunscreen use (yes)	−0.145	−2.841	2.550	0.915
Age (years)	−0.067	−0.125	−0.006	0.325
Waist circumference (cm)	−0.010	−0.116	0.096	0.857
Body fat (%)	0.083	−0.072	0.237	0.291
BMI (kg/m^2)	−0.030	−0.324	0.264	0.842
HDL-c (mg/dL)	0.008	−0.106	0.122	0.886
LDL-c (mg/dL)	−0.017	−0.060	0.027	0.446
Triglycerides (mg/dL)	−0.006	−0.023	0.011	0.468
Free fatty acids (nmol/mL)	−1.284	−7.374	4.807	0.675
Glucose (mg/dL)	0.043	−0.100	0.186	0.552

[a] Statistical test: Student's *t*-test ($p < 0.05$). 95% confidence interval (95% CI).

Table 4
Multivariate linear regression model for subjects with an s25(OH)D_3 concentration < 30 ng/mL (subjects with calcidiol serum levels below 30 ng/mL).

	β Coefficient	95% CI		p
s25(OH)D_3 < 30 ng/mL model				
*$p < 0.05$**				
Age (years)	−0.076	−0.141	−0.010	0.02*
BMI (kg/m^2)	−0.213	−0.376	−0.050	0.01*
HDL-c (mg/dL)	−0.104	−0.159	−0.049	<0.001*
Glucose (mg/dL)	0.108	0.015	0.202	0.02*
Sun exposure (indoor work environment)	−2.593	−3.933	−1.253	<0.001*

Statistical test: Multivariate regression model using significant variables for the serum 25(OH)D_3 < 30 ng/mL group, wherein the 95% CI means the 95% confidence interval and p is the p-value of the model variable.
* Statistically significant difference ($p < 0.05$).

Pearson's correlation analysis and an analysis using scatter plots were performed with the variables used in the multivariate regression analysis. The variables age ($r = -0.131$, $p < 0.05$), BMI ($r = -0.080$, $p < 0.05$), HDL-c ($r = 0{,}229$, $p < 0.001$) and sun exposure (indoor work environment) ($r = -0.199$, $p = 0.05$) were significant (Fig. 1) for the s25(OH)D_3 < 30 ng/mL model.

3.2. In vitro study

3.2.1. ADSC differentiation

The culture of undifferentiated cells and the confirmation of adipogenic, osteogenic and chondrogenic differentiation are shown in Fig. 2a–d, respectively. The accumulation of lipids stained with Oil red O is shown in Fig. 2b, whereas calcium deposits stained with Alizarin Red are shown in Fig. 2c. Conversely, the glycosaminoglycan-rich extracellular matrix stained with Alcian Blue is shown in Fig. 2d.

3.2.2. MTT assay

The MTT assay for cell viability showed the following results for the tested concentrations (15.625, 31.25, 62.5, 125 and 250 nM): 93.90 ± 1.91, 93.92 ± 2.77, 87.20 ± 2.22, 52.74 ± 9.94 and 24.70 ± 7.03 in the 24-h period; 91.80 ± 4.52, 88.53 ± 2.98, 75.09 ± 5.77, 45.10 ± 11.48, 24.46 ± 7.50 in the 48-h period and 87.11 ± 3.82, 83.79 ± 2.26, 66.22 ± 4.87, 34.61 ± 8.91 and 16.83 ± 3.32 in the 72-h period, respectively (Fig. 3a). The statistical analysis showed no significant differences in cell viability between treatment with the 15.625 nM and 31.25 nM 1,25$(OH)_2D_3$ concentrations and the control ($p > 0.05$) at any treatment time point. The 62.5 nM 1,25$(OH)_2D_3$ concentration significantly decreased ($p < 0.05$) the cell viability at 48 and 72 h. Moreover, the 125 and 250 nM 1,25$(OH)_2D_3$ concentrations had significant effects ($p < 0.05$) on cell viability at all treatment time points (b).

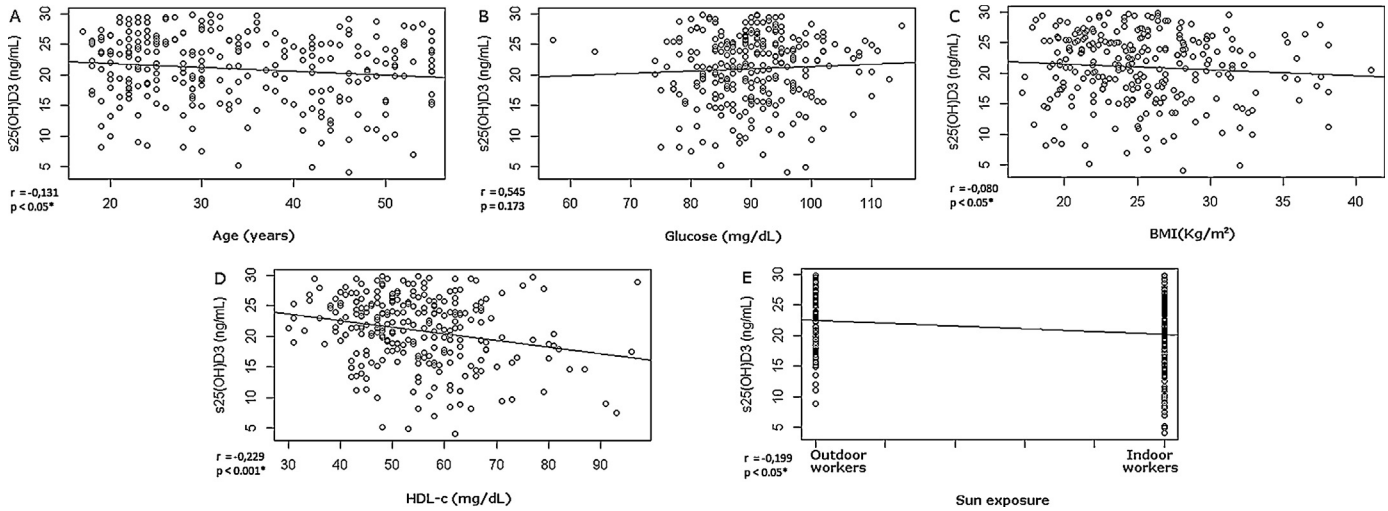

Fig. 1. Scatter plot and correlation coefficients of the variables used in the s25(OH)D_3 < 30 ng/mL group multivariate regression analysis. a) Age (years; $r = -0.131$, $p < 0.05$), b) glucose (mg/dL; r = 0.545, p = 0.173), c) BMI (kg/m^2; $r = -0.080$, $p < 0.05$), d) HDL-c (mg/dL; $r = 0.229$, p < 0.001) and sun exposure (work environment; $r = -0.199$, $p = 0.05$).

3.2.3. IC50 calculation

The calculated IC50 of 1,25$(OH)_2D_3$ for the 24-h treatment was 138.97 nM. A qualitative evaluation was performed for the same concentration and treatment time. The number of cells per field of view decreased, cytoplasmic extensions were observed, the appearance was no longer fibroblastoid, and adhesiveness occurred; these changes caused the cells to adopt a rounded appearance (Fig. 4).

4. Discussion

The present study detected hypovitaminosis D in a sample of adult individuals from southern Brazil. The same finding was also reported by other population studies conducted in cities of the southeast, central-west and north regions [24,27,57–59], indicating that this public health problem is not limited to a specific Brazilian demographic region.

In Brazil, the consumption of vitamin D-rich foods is considered low [27] and is characterised as inadequate throughout the country according to the Brazilian Family Budget Survey (Pesquisa Brasileira de Orçamento Familiar) [60]. This inadequate consumption affects several age groups (10 to 13, 14 to 18, 19 to 59 and 60 years and older) and both genders. Furthermore, rare sun exposure and the sedentary lifestyle typical of the study populations [61,62] aggravate the condition, even though Brazil is a tropical country with a high solar incidence for most of the year. Brazilian population studies have shown weight gain and confirmed the prevalence of hypovitaminosis D [24,63].

The two key determinants of s25(OH)D3 are sun exposure and the body fat percentage according to Bolland, Grey, Ames, Mason, Horne, Gamble and Reid [64]. Therefore, sun exposure should ideally occur at least three times a week for 5 to 15 min from 10 AM to 3 PM. This exposure time would suffice for vitamin D production under ideal conditions [65]. We should emphasise that moderate sunscreen use has no effect on the conversion of 7-dehydrocholesterol into vitamin D_3 [66]. However, because an increase in sun exposure tends to be insufficient in the presence of a high percentage of body fat, supplementation is required under these circumstances [67]. The negative association between s25(OH)D_3 and body fat may be attributed to the sequestration of fat-soluble, skin-generated or supplemented vitamin D in adipocytes before the transport of this molecule to the liver for conversion into 25(OH)D_3 [24]. Therefore, we can use these facts to explain the results from this research study, which showed hypovitaminosis D in 79.23% of the sample.

The univariate data analysis showed that only the 25(OH)D_3 and LDL-c levels were significantly different between the groups. Vitamin D deficiency affects various metabolic factors of the body, including dyslipidaemia, and thus provides a justification for testing the correlation between vitamin D and the development of cardiovascular diseases [68–71]. Furthermore, Yin, Sun, Zhang, Lu, Sun, Cui and Wang [72] reported that LDL-c might be the most important of the risk factors that increase the risk for cardiovascular diseases. Thus, this dyslipidaemic factor must be studied to improve the quality of life of the community.

Circulating LDL-c may be oxidised by free radicals, triggering the inflammatory process in blood vessels and thereby recruiting macrophages, which absorb LDL-c particles. Due to their inability to hydrolyse LDL-c, macrophages become foam cells, which are deposited and agglomerate in blood vessel walls and cause atheroma plaques [73]. The main mechanism of LDL-c removal is known as reverse cholesterol transport or cholesterol efflux and occurs when the endogenous cholesterol of peripheral cells and macrophages is removed by binding to HDL-c particles, thereby reducing the atheromatous plaques [74]. The fat-soluble nature of vitamin D leads to a correlation between LDL-c, HDL-c and vitamin D, suggesting that LDL-c removal may be mediated by vitamin D because macrophages have the vitamin D receptor (VDR) [75]. Thus, vitamin D plays a key role in regulating reverse cholesterol transport [76], modulating genes related to the increase in the cholesterol efflux, including *ABCA1* [77] and others [78], and inducing the polarisation of anti-inflammatory macrophages, thereby partially limiting the formation of atheromatous plaques [79–81].

The mean HDL-c concentration of the study population was almost identical to and within the levels recommended by the American Society of Cardiology [82] and was close to the values considered optimal (HDL-c ≥ 60 ng/mL). Conversely, the population with s25(OH)D_3 < 30 ng/mL showed a significant negative correlation between vitamin D and HDL-c. This finding indicated that vitamin D failed to modulate the HDL-c levels in the present research study. Although most studies showed opposite results

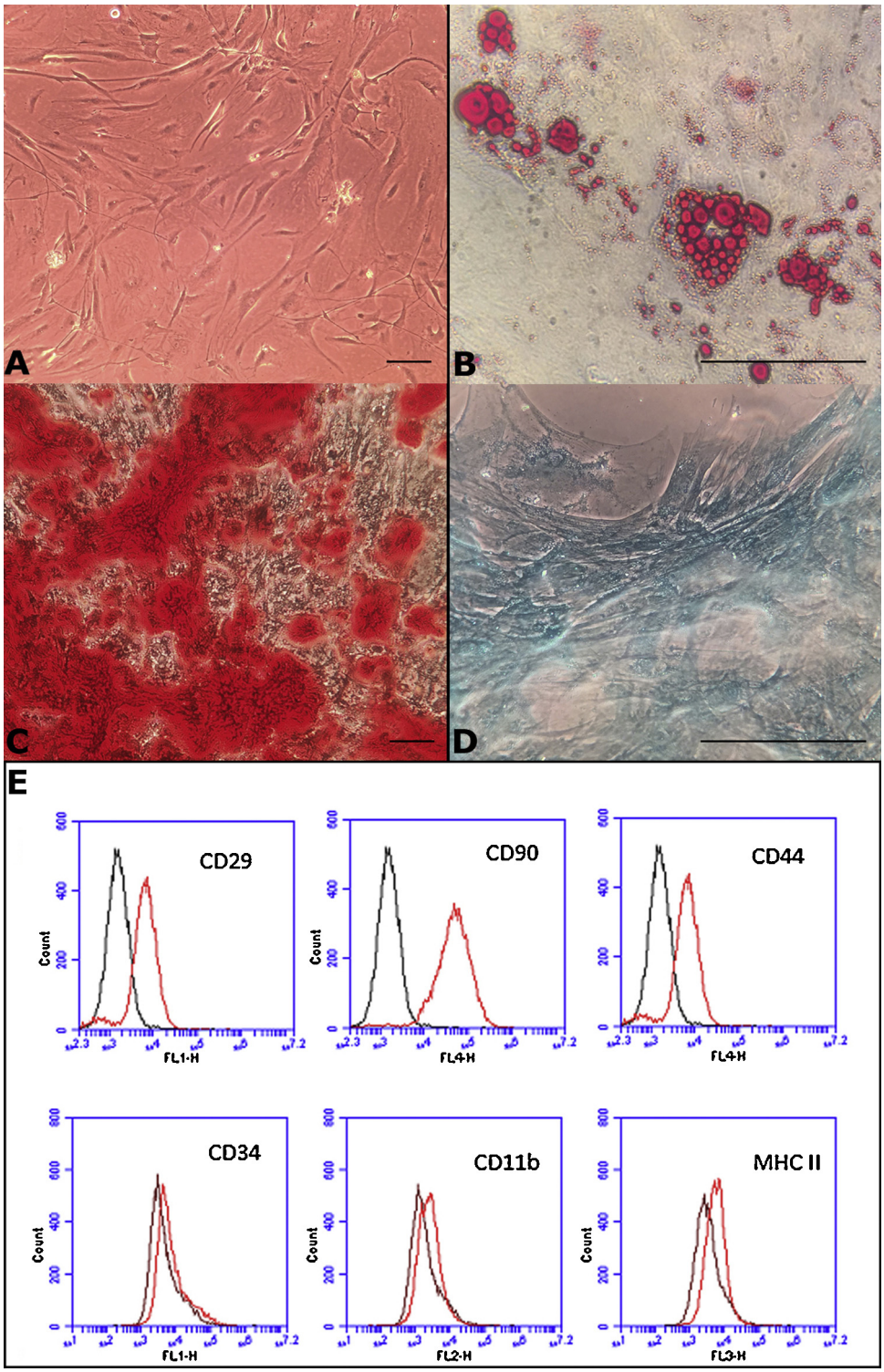

Fig. 2. Morphology, characterization and differentiation potential of mesenchymal stem cells derived from human adipose tissue. a) Undifferentiated culture demonstrating cells with fibroblast characteristics, b) adipogenic differentiation culture and lipid vacuoles stained with Oil red O, c) osteogenic differentiation culture and calcium deposits stained with Alizarin Red and d) chondrogenic differentiation culture and glycosaminoglycan-rich extracellular matrix stained with Alcian Blue. Bars represent 50 μm. e) Immunophenotypic characterization graphs demonstrating that the cells are positive for the CD29, CD90 and CD44 molecular markers but negative for CD34, CD11b and MHC II.

from this finding [7,83,84], some studies, including vitamin D supplementation studies, showed no increase [85] and/or a decrease in the HDL-c profile [86–88]; thus, the vitamin D and HDL interaction has not been completely elucidated. Other variables of our study, including triglycerides, free fatty acids and glucose, showed no significant results in the univariate regression analysis. However, dyslipidaemia and hyperglycaemia were found in obese or overweight patients [33], and the modulating potential of vitamin D in adipogenesis was previously examined [89].

In the present study, the general data analysis and literature review clearly established the associations between s25(OH)D_3

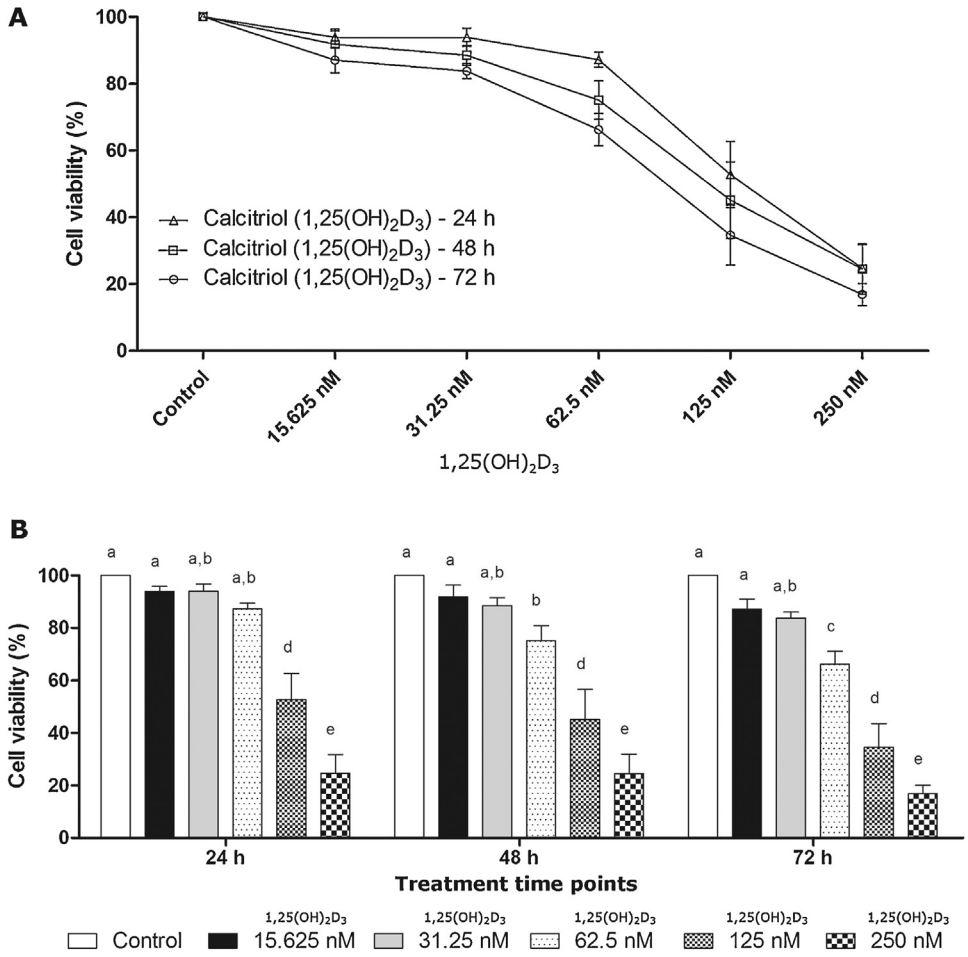

Fig. 3. Graphs representing the results obtained from the MTT assay. a) Mean ± SEM of the cell viability of ADSCs treated with calcitriol ($1,25(OH)_2D_3$) over different treatment time periods (24, 48 and 72 h). The following results were obtained for the tested concentrations (15.625, 31.25, 62.5, 125 and 250 nM): 93.90 ± 1.91, 93.92 ± 2.77, 87.20 ± 2.22, 52.74 ± 9.94 and 24.70 ± 7.03 in the 24-h period; 91.80 ± 4.52, 88.53 ± 2.98, 75.09 ± 5.77, 45.10 ± 11.48, 24.46 ± 7.50 in the 48-h period and 87.11 ± 3.82, 83.79 ± 2.26, 66.22 ± 4.87, 34.61 ± 8.91 and 16.83 ± 3.32 in the 72-h period, respectively. b) Comparison of the mean cell viability between the different treatment times with calcitriol ($1,25(OH)_2D_3$; 24, 48 and 72 h). Different letters indicate significant differences (ANOVA/Bonferroni; $p < 0.05$). No significant difference was observed in cell viability between treatment with the 15.625 nM and 31.25 nM $1,25(OH)_2D_3$ concentration and the control ($p > 0.05$) at any treatment time point. The 62.5 nM $1,25(OH)_2D_3$ concentration significantly decreased ($p < 0.05$) the cell viability at 48 and 72 h, and the 125 and 250 nM $1,25(OH)_2D_3$ concentrations had significant effects ($p < 0.05$) on cell viability at all treatment time points.

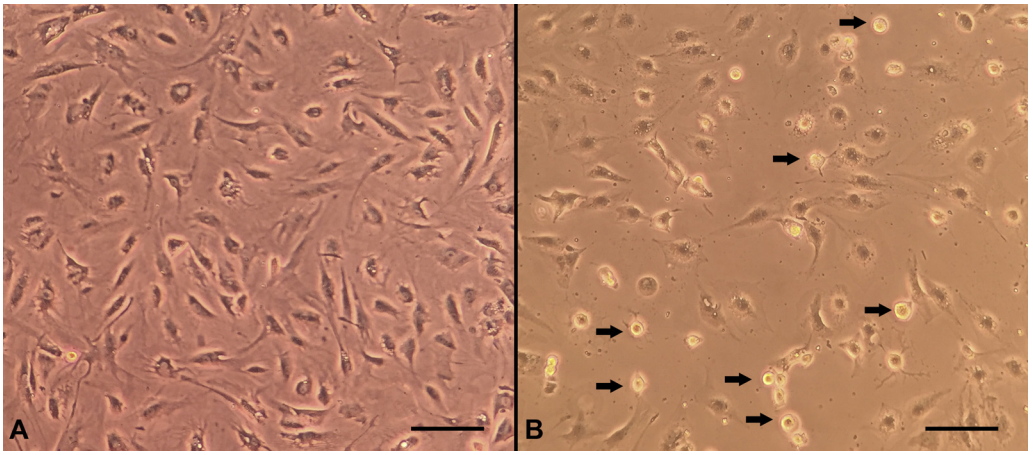

Fig. 4. Photomicrograph of ADSCs. a) Culture in HDMEM and normal morphology. b) Culture in HDMEM with 138.97 nM calcitriol ($1,25(OH)_2D_3$) for 24 h. Arrows indicate detached cells with the loss of the fibroblastoid appearance. Bars represent 50 μm.

deficiency, various anthropometric and biochemical variables and metabolic disorders, including obesity-related diseases. No study variable was a significant predictor of the s25(OH)D_3 concentration $\geq$ 30 ng/mL. Conversely, BMI, age, HDL-c, glucose and sun exposure were significant predictors of the s25(OH)D_3 concentration $<$ 30 ng/mL.

Obesity affects approximately 13% of the global population [90]. In Brazil, obesity affects almost one in five Brazilians according to the Ministry of Health (Ministério da Saúde, Brasil) [63]. These data are worrying and place a burden on public health spending worldwide. Additionally, the Brazilian Unified Health System (Sistema Único de Saúde–SUS) spends approximately 150 million US dollars treating obesity-related diseases [91].

The body fat percentage and BMI are two key analysis parameters for obesity, and both parameters have been reported to be inversely correlated with obesity [92,93]. Our results also support this inference and increase interest in a better understanding of the cellular biology of obesity and its correlation with vitamin D levels. However, the relevance of vitamin D and the differentiation and/or cell death process to the biology of obesity is a key factor that requires further study [43]. The current understanding indicates that obesity results from a combination of three key events: (I) adipocyte hypertrophy; (II) hyperplasia of ADSCs and (III) the balance between the cell differentiation process (stem cells into adipocytes) and the cytostatic and/or cytotoxic effects on ADSCs, pre-adipocytes or adipocytes themselves [34]. Using an *in vitro* test system, our study showed that the viability of ADSCs depended on the time and concentration used for vitamin D supplementation (i.e., the viability of ADSCs was affected by the vitamin D concentration and exposure time). The IC50, which was between the two highest tested 1,25$(OH)_2D_3$ concentrations, was also calculated based on those results.

The MTT assay is commonly used to evaluate cell viability primarily through cytotoxic evaluation of the tested compound. Our results corroborate and complete the findings of Valle, Almalki and Agrawal [94]. These authors examined the cytotoxicity of 1,25$(OH)_2D_3$ by testing the release of the enzyme glucose-6-phosphate dehydrogenase in porcine ADSCs at a 100 nM concentration to assess cytotoxicity and necrosis at that tested concentration. Thus, the experimental data reported herein may promote the study of new anti-obesity treatments considering 1,25$(OH)_2D_3$ supplementation and the biology of obesity, particularly the cytotoxic effects on cells that cause adipose tissue hyperplasia.

5. Conclusions

Given the above findings, the present study suggests that hypovitaminosis D is prevalent in the southern Brazilian study population and establishes a correlation between a low serum vitamin D concentration and increased LDL-c, low sun exposure (indoor work environment) and increased body fat. These findings suggest a key association between vitamin D deficiency and the development of obesity. Furthermore, the *in vitro* test performed in this study indicates that vitamin D supplementation may be a strategy for the development of anti-obesity treatments because this vitamin induces cell death (i.e., has a cytotoxic effect on ADSCs), which may be responsible for adipose tissue hyperplasia.

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