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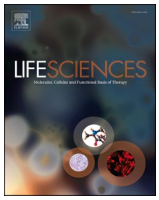

Differential effects of dolutegravir, bictegravir and raltegravir in adipokines and inflammation markers on human adipocytes

Pere Domingo [a,b,1], Tania Quesada-López [a,b,c,d,e,f,1], Joan Villarroya [c,d,e,f], Montserrat Cairó [c,d,e,f], Maria Del Mar Gutierrez [a,b], Maria Gracia Mateo [a,b], Isabel Mur [a,b], Noemí Corbacho [a,b], Joan Carles Domingo [c,d], Francesc Villarroya [c,d,e,f], Marta Giralt [c,d,e,f,*]

[a] Department of Infectious Diseases, Hospital de la Santa Creu i Sant Pau, Barcelona, Catalonia, Spain
[b] Institut de Recerca de l'Hospital de la Santa Creu i Sant Pau, Barcelona, Catalonia, Spain
[c] Departament de Bioquímica i Biomedicina Molecular, Facultat de Biologia, Universitat de Barcelona, Barcelona, Catalonia, Spain
[d] Institut de Biomedicina (IBUB), Universitat de Barcelona, Barcelona, Catalonia, Spain
[e] CIBER Fisiopatología de la Obesidad y Nutrición (CIBEROBN), Instituto de Salud Carlos III, Madrid, Spain
[f] Institut de Recerca Sant Joan de Déu (IRSJD), Esplugues de Llobregat, Catalonia, Spain

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ABSTRACT

Aims: To assess the potential direct effects of the integrase strand-transfer inhibitors (INsTIs) dolutegravir, bictegravir, and raltegravir, drugs used as treatment for people living with human immunodeficiency virus (PLWH), on human adipose cells.

Main methods: Drugs were added to the differentiation medium of human Simpson-Golabi-Behmel syndrome (SGBS) adipose cells and morphological adipogenesis was monitored for 10 days. Also, adipocytes were exposed to drugs following differentiation (day 14). The gene expression levels of selected adipogenesis markers, adipocyte metabolism markers, adipokines, and cytokines were determined by quantitative-reverse transcription polymerase-chain reaction. The release of adiponectin and leptin into the culture medium was measured using specific enzyme-linked immunosorbent assay, and release of interleukin-6 and chemokine (C—C motif) ligand-2 using Multiplex assays.

Key findings: Overall morphological adipogenesis was unaltered by INsTIs. The expression of adipogenesis marker genes (peroxisome proliferator-activated receptor-ɣ and lipoprotein lipase) was slightly reduced in dolutegravir-treated differentiating adipocytes. Bictegravir repressed gene expression and the release of pro-inflammatory cytokines in differentiating adipocytes. Dolutegravir and raltegravir increased interleukin-6 gene expression, but only dolutegravir increased interleukin-6 release. Dolutegravir repressed adiponectin expression and release in differentiating adipocytes and had a similar but milder effect on leptin. Drug treatment of mature adipocytes reduced adiponectin gene expression in response to dolutegravir.

Significance: The INsTIs studied do not have a significant effect on human adipose cell differentiation but exert distinct effects on gene expression and secretion of adipokines and cytokines. These findings will help understand and manage the effects of INsTI-containing treatments on body weight and metabolic dysregulation in PLWH.

Abbreviations: INsTIs, integrase strand-transfer inhibitors; HIV, human immunodeficiency virus; PLWH, people living with HIV; SGBS, Simpson-Golabi-Behmel syndrome; PPAR, peroxisome proliferator-activated receptor; LPL, Lipoprotein lipase; TNF, tumor necrosis factor; CCL2, Chemokine (C—C motif) ligand 2; MCP-1, Monocyte chemoattractant protein-1; IL6, Interleukin-6; DTG, dolutegravir; BIC, bictegravir; RAL, raltegravir; FBS, fetal bovine serum; qRT-PCR, Quantitative Reverse Transcription Polymerase-Chain Reaction..

* Corresponding author at: Departament de Bioquímica i Biomedicina Molecular, Facultat de Biologia, Universitat de Barcelona, Diagonal, 643, 08028 Barcelona, Spain.
E-mail address: mgiralt@ub.edu (M. Giralt).
[1] These authors contributed equally to this work.

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1. Introduction

Weight gain and increased adiposity are increasingly found in people living with human immunodeficiency virus, HIV (PLWH), contributing to an increased risk of serious medical complications. Recent studies have raised concerns regarding excess weight gain and metabolic abnormalities specifically associated with the use of integrase strand-transfer inhibitors (INsTIs) for the treatment of PLWH. The first warning came from observational studies, and connections with INsTI treatments were later confirmed in randomized clinical trials [1–3]. However, the pathophysiological bases of the effects of INsTIs on adiposity and the exact contribution of each type of INsTI drug remain largely unknown [3,4].

Increased body weight, as in obesity, is associated with the enlargement of adipose tissue. Adipocytes are key sites of fuel metabolism storage (in the form of triglycerides), and energy release in the form of free fatty acids in periods of energy demand (such as fasting). Additionally, adipocytes are responsible for producing circulating adipokines that influence systemic metabolism [5]. Increased adiposity is characterized by the enlargement (hypertrophy) of adipocytes and, sometimes, even hyperplasic phenomena. Abnormally enlarged adipose tissue in obesity is associated with systemic metabolic alterations, mainly hyperglycemia, insulin resistance, and dyslipidemia. Concomitant with the metabolic alterations in the adipose depots, disturbances of adipokines (such as leptin, adiponectin) and cytokines (such as pro-inflammatory tumor necrosis factor-alpha (TNFα) and chemokine (C—C motif) ligand 2 (CCL2)) secretion by adipose tissue greatly contribute to systemic metabolic alterations in overweight and obese people [5]. Such conditions have been increasing in PLWH.

Studies on the effects of anti-retroviral drugs on human adipose cells can provide pre-clinical information regarding the potential of these drugs to alter adipose tissue. Likewise, these pre-clinical approaches have proven useful for identifying molecular targets involved in the metabolic side-effects of first-generation anti-retroviral drugs namely protease inhibitors and reverse transcriptase inhibitors [6–12]. The capacity of the distinct INsTIs currently used for the treatment of PLWH suspected to promote adiposity to directly affect human adipose cells is poorly understood. Thus, the goal of the present study was to assess the potential direct effects of the INsTIs, dolutegravir (DTG), bictegravir (BIC), and raltegravir (RAL), on adipogenic differentiation, relevant gene expression, and the release of cytokines and adipokines by human adipose cells.

2. Materials and methods

2.1. Reagents

Chemicals and reagents for cell culture were purchased from Sigma-Aldrich (St Louis, Missouri, USA). Rosiglitazone which was purchased from Alexis Biochemicals (Enzo Life Sciences, Farmingdale, New York, USA). DMEM/F12 medium and fetal bovine serum (FBS) were purchased from Life Technologies (Carlsbad, California, USA). DTG and RAL were obtained from MedChemExpress (Monmouth Junction, NJ 08852, USA); and BIC was provided by Gilead (Foster City, California, USA). For the initial assessments of the effects of anti-retroviral drugs before concentration-response analyses, the drugs were used at the highest concentrations that did not cause cytotoxicity in specific cell systems. The drugs were dissolved using dimethyl sulfoxide (DMSO) as the vehicle. Controls included amounts of DMSO (≤0.1 % DMSO of total cell medium volume) equal to that used in the drug-treated cells.

2.2. Human adipocyte cell culture

To assess the effects of INsTIs on adipogenesis, we used human Simpson-Golabi-Behmel syndrome (SGBS) pre-adipocytes. This cell strain has been validated as a model of human adipogenic differentiation [13] and has been extensively used to assess the effects of anti-retroviral drugs on human adipocyte function [14–17]. Human SGBS pre-adipocytes were cultured and differentiated into adipocytes, as previously reported [14,18]. Briefly, SGBS pre-adipocytes were maintained in DMEM/F12 containing 10 % FBS. After the cells reached confluency, adipogenic differentiation was initiated by first incubating the cells for seven days in a serum-free medium containing 20 nM insulin, 100 μg/ml human apo-transferrin, 0.2 nM triiodothyronine (T3), and 100 nM cortisol, supplemented with 25 nM dexamethasone, 500 μM 3-isobutyl-methylxanthine, and, 2 μM rosiglitazone to obtain the fully differentiated adipocytes or 0.5 μM rosiglitazone (a sub-optimal concentration for adipogenic differentiation) for chronic treatments. The cells were subsequently switched to an adipogenic differentiation medium containing 20 nM insulin, 100 μg/ml human apo-transferrin, 0.2 nM triiodothyronine (T3), and 100 nM cortisol only and maintained for another three days (in the case of chronic treatments) or seven days for treatments in fully differentiated adipocytes (where, >90 % of the cells had acquired a differentiated adipocyte morphology, as evidenced by lipid droplet accumulation). Depending on the experimental design, the cells were treated with drugs throughout the 10-day differentiation process or acutely for 24 h once they were fully differentiated (14 days after the induction of differentiation).

2.3. Assessment of lipid accumulation in adipocytes

To assess the effects of these drugs (dolutegravir, raltegravir, and bictegravir) on adipocyte differentiation and lipid accumulation, micrographs of the cell cultures on day 10 after the induction of differentiation were captured using a Motic AE2000 microscope with a Moticam 5.0 MP camera (Motic Instruments INC, Richmond, BC, Canada). Micrographs, as jpg files, were imported into the ImageJ software (Wayne Rasband at the National Institutes of Health (NIH)). The image type was processed into an RGB stack (Image > Type > RGB-Stack) and a montage of the three channels was performed (Image > Stacks > Make Montage). Once in the RGB stack, the threshold of the image was adjusted (lower threshold of 160 and an upper threshold of 250) (Image > Adjust > Threshold). Area, area fraction, limit to threshold, and display label parameters were set for measurement (Analyze > Set Measurements). Image measurements were then performed (Analyze > Measure). The measurements obtained were subsequently used for comparison.

2.4. RNA isolation, conventional reverse transcription, and quantitative reverse transcription polymerase-chain reaction (qRT-PCR)

Total RNA was purified from cells using an affinity column-based method (Macherey-Nagel; Thermo Fisher Scientific, Waltham, Massachusetts, USA) as previously described [14]. Reverse transcription was performed in a total volume of 20 μl using random hexamer primers (Applied Biosystems; Waltham, Massachusetts, USA) and 0.5 μg total RNA. PCR was performed on an ABI/Prism 7700 sequence detector system using 20 μl of a reaction mixture containing 1 μl of cDNA, 10 μl TaqMan Universal PCR master mix, 250 nM probes, and 900 nM primers from an Assays-on-Demand gene expression assay mix (TaqMan; Applied Biosystems; Waltham, Massachusetts, USA). The following Assay-on-Demand probes from Life Technologies were used: Adiponectin, Hs00605917; Chemokine (C—C motif) ligand 2 (CCL2), Hs00234140; Cytochrome-c oxidase subunit IV (COX4I1), Hs00266371; Interleukin-6 (IL6), Hs00174131; Leptin, Hs00174877; Lipoprotein lipase (LPL), Hs00173425; Peroxisome proliferator-activated receptor-ɣ (PPARɣ), Hs00231674; Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PPARGC1A), Hs00173304_m1; Solute carrier family 2, facilitated glucose transporter member 1 (GLUT1, Slc2a1), Hs00892681_m1; Solute carrier family 2, facilitated glucose transporter member 4 (GLUT4, Slc2a4), Hs99999901; RPLP0, Hs99999902. Controls without RNA, primers, or reverse transcriptase

Table 1
Effects of dolutegravir (DTG), raltegravir (RAL) and bictegravir (BIC) on SGBS human adipogenic differentiation.

Drug concentration (μM)	0.01	0.1	1	10
Dolutegravir	99.6 ± 2.3	94.8 ± 3.7	104.8 ± 1.3	109.5 ± 0.2
Raltegravir	108.7 ± 0.6	100.1 ± 0.1	106.8 ± 3.5	104.0 ± 1.8
Bictegravir	106.9 ± 0.3	101.8 ± 2.6	109.4 ± 1.2	109.1 ± 0.6

were included in each set of experiments. The relative amounts of individual mRNAs were calculated using the comparative ($2^{-\Delta CT}$) method and normalized to that of the reference control gene (RPLP0 mRNA) according to the manufacturer's instructions. Each sample was run in duplicates, and the mean value of duplicates was used in the calculations. Parallel calculations performed using other reference control genes (18S rRNA) yielded essentially the same results.

2.5. Cytotoxicity assays

Cytotoxicity was determined using a CytoTox96 kit (Promega; Madison, Wisconsin, USA), following the manufacturer's instructions. Briefly, cytotoxicity was calculated as the lactate dehydrogenase activity present in the cell culture medium relative to the maximum activity observed after total cell lysis of cells after exposure to drugs for 24 h.

2.6. Protein analysis

Adiponectin, leptin, IL6, and CCL2 (also referred to as monocyte chemoattractant protein-1, MCP-1) were quantified using medium collected from the last five days of adipocyte culture, before harvest. ELISA kits specific for human adiponectin (EZHADP-61 K, Merck Life Science; Madison, Wisconsin, USA) and leptin (DLP00, R &D Systems; Minneapolis, Minnesota, USA), were used for quantification. IL6 and CCL2 levels were quantified using a multiplex analysis system (Lynco Research/Merck; Darmstadt, Germany) and a Luminex 100ISv2 instrument (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

2.7. Statistical analyses

Statistical analyses were performed using GraphPad Prism 9 Software (San Diego, California USA) using one-way ANOVA and Dunnett's multiple comparison test. Significance is indicated in the text (the threshold value to determine significance was set to $P \leq 0.05$).

3. Results

3.1. Pre-adipocytes treatment with INsTIs does not elicit cytotoxicity

The effects of DTG, RAL, and BIC on the adipogenic differentiation of human SGBS pre-adipocytes were tested over a concentration range of 0.01 to 10 μM, which included the C_{min} and C_{max} circulating concentrations as previously reported in treated patients [19,20]. Differentiating pre-adipocytes were exposed to DTG, BIC, or RAL (0.01 μM–10 μM) for 24 h, and the potential cytotoxicity of these drugs at the different

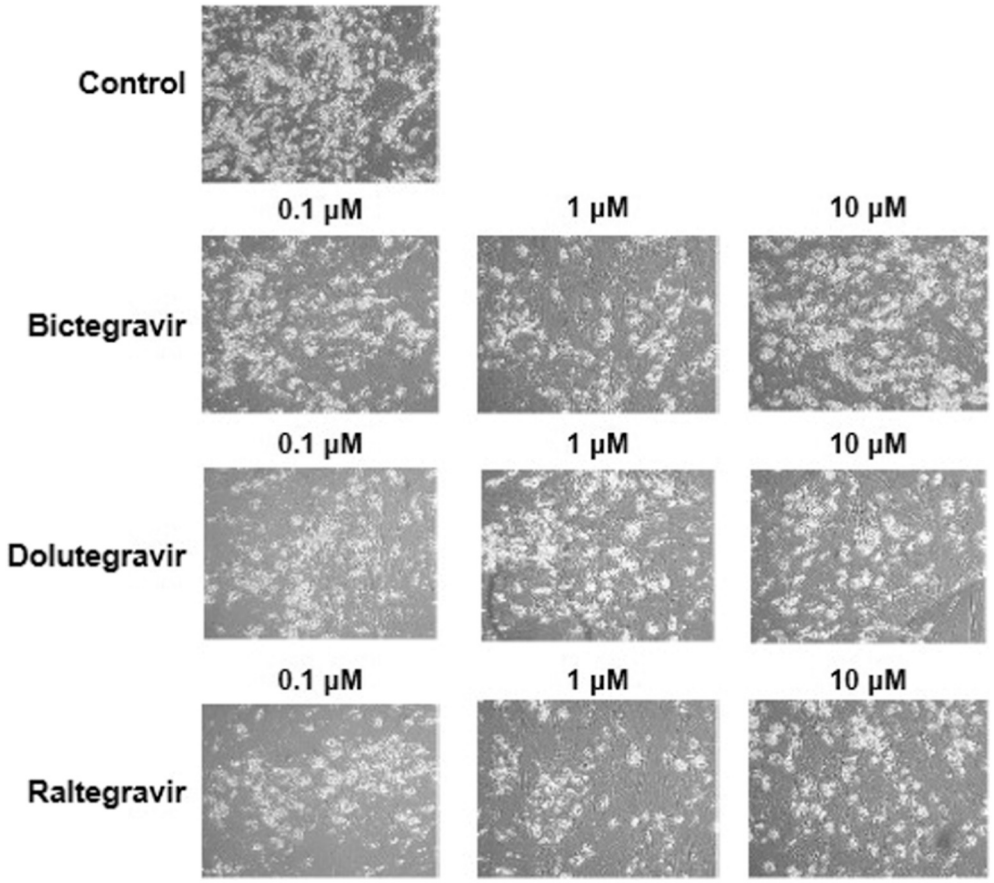

Fig. 1. Effects of dolutegravir, raltegravir and bictegravir on adipogenic differentiation of SGBS human pre-adipocytes in culture. SGBS human pre-adipocytes were differentiated in culture in the presence of the indicated concentrations of drugs. Treatment was initiated on day 0 and continued throughout the differentiation process (10 days). Representative micrographs of adipocyte cell cultures differentiating in the presence of the indicated concentrations of drugs are shown.

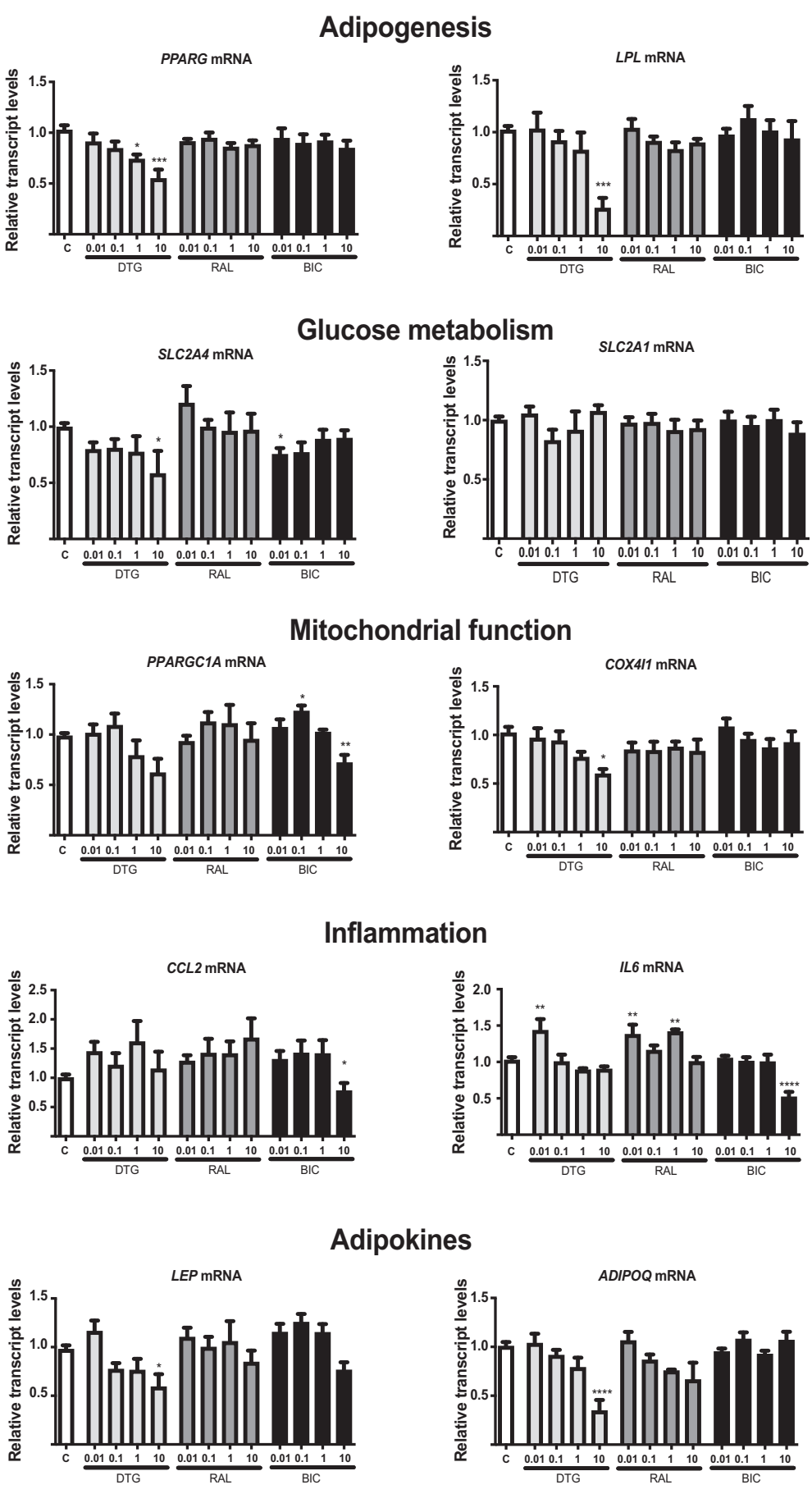

Fig. 2. Effects of dolutegravir (DTG), raltegravir (RAL), and bictegravir (BIC) on the expression levels of genes related to adipogenic function, inflammation, mitochondrial function, and adipokines in SGBS human adipocytes differentiating in culture. SGBS human pre-adipocytes were differentiated in culture in the presence of the indicated concentrations of drugs. Data on mRNA levels are presented as means ± SEM from 3 to 4 independent cell culture experiments, each sample analyzed in duplicate, and are expressed relative to values from untreated control cells (defined as 1). Statistical analyses were performed using one-way ANOVA and Dunnett's multiple comparison test. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001 for each drug treatment vs. control.

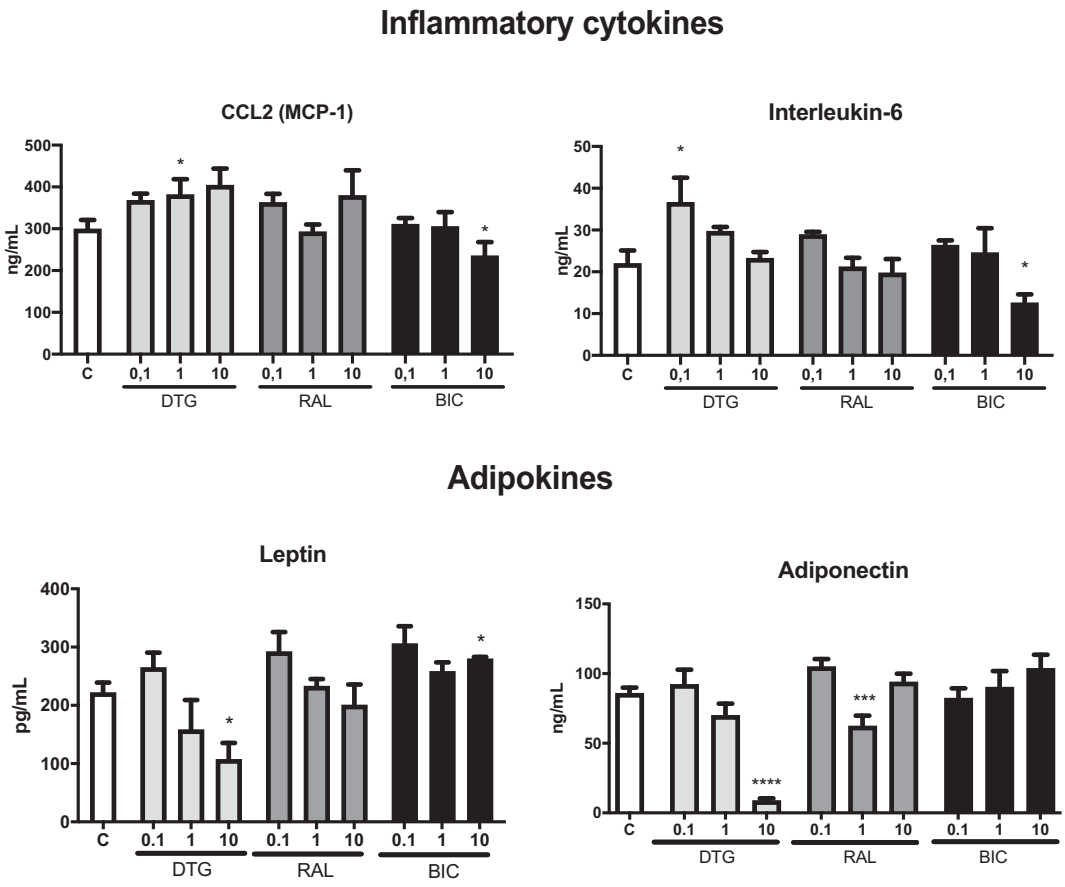

Fig. 3. Effects of dolutegravir (DTG), raltegravir (RAL), and bictegravir (BIC) on the release of CCL2 (MCP-1), interleukin-6, leptin, and adiponectin to the cell culture medium of differentiating SGBS human adipocytes. SGBS human pre-adipocytes were differentiated in culture in the presence of the indicated concentrations of drugs. Data corresponds to concentrations accumulating in the cell culture medium in the last 5 days of culture. Data are means ± SEM from 3 to 4 independent cell culture experiments, and each sample was analyzed in duplicate. Statistical analyses were performed using one-way ANOVA and Dunnett's multiple comparison test. *P < 0.05, ***P < 0.001, and ****P < 0.0001 for each drug treatment vs. control.

concentrations was estimated. No significant cytotoxicity was observed in the cell cultures exposed to any of the tested concentrations of DTG, BIC, or RAL (Supplemental Table 1). This is consistent with previous reports, in which DTG and BIC exhibited significant cytotoxicity only at higher concentrations (>10 μM) in other cell types (such as hepatocytes, fibroblasts, human prostate cells, and hepatocarcinoma cells) [21]. RAL showed no cytotoxicity at concentrations up to 10 μM in SGBS pre-adipose cells [14,18].

3.2. INsTIs did not alter overall adipogenesis but have differential effects on adipocyte gene expression

To study the effects of each drug on adipogenic differentiation, INsTIs treatments was initiated on day zero and continued throughout the differentiation process (up to 10 days, as described in the previous section). The effects of the drugs on the morphological differentiation of adipocytes were determined using a phase-contrast microscope and Image J software to quantify the cell culture surface occupied by differentiated (lipid droplet-containing) cells (see Materials and Methods section). Data are expressed as percentages relative to control cultures (Table 1). None of the tested drugs had a major effect on the acquisition of an adipocyte morphology, as drug-treated cells consistently exhibited unaltered lipid accumulation relative to the control (Fig. 1 and Table 1).

Data are expressed as mean ± SEM of the percent of lipid accumulation relative to the 100 % adipogenesis elicited by SGBS cultured under control (sub-optimal 0.5 μM rosiglitazone-induced adipogenesis) adipogenic conditions, as determined by performing phase-contrast microscopic imaging and using the Image J software to quantify the cell culture surface occupied by differentiated (lipid droplet-containing) cells. None of the results was significantly different from control values (one-way ANOVA Dunnett's multiple comparisons test).

Gene expression was quantified after 10 days of the adipogenic differentiation induction in the presence of INsTIs (Fig. 2). We observed little alteration in response to RAL (only a minor induction of IL6 mRNA levels), consistent with a previous report [14]. BIC did not alter the expression levels of genes encoding adipogenic molecular factors (peroxisome proliferator-activated receptor-gamma, PPARγ; lipoprotein lipase, LPL), glucose transporter-4 (SLC2A4), glucose transporter-1 (SLC2A1), or adipokines (leptin, LEP, and adiponectin, ADIPOQ). However, BIC significantly reduced the expression levels of genes encoding the pro-inflammatory cytokines, CCL2 (MCP-1) and interleukin-6 (IL6), at the highest concentration tested (10 μM). DTG applied at 10 μM significantly reduced the gene expression levels of PPARγ (the master gene regulator of adipogenesis), lipoprotein lipase, leptin, and adiponectin (Fig. 2). Considering the release of leptin and adiponectin to the adipocyte culture medium, the highest doses of DTG mildly reduced the level of released leptin and dramatically decreased that of adiponectin; BIC did not alter the release of leptin or adiponectin; and RAL had minor effects on these parameters (Fig. 3). DTG significantly ($P \leq 0.05$) increased the release of CCL2 (MCP-1) and IL6 into the cell culture medium, whereas BIC reduced the release of both cytokines in differentiating adipocytes treated at 10 μM (Fig. 3). RAL had minor effects on these parameters. The induction of IL6 mRNA expression was

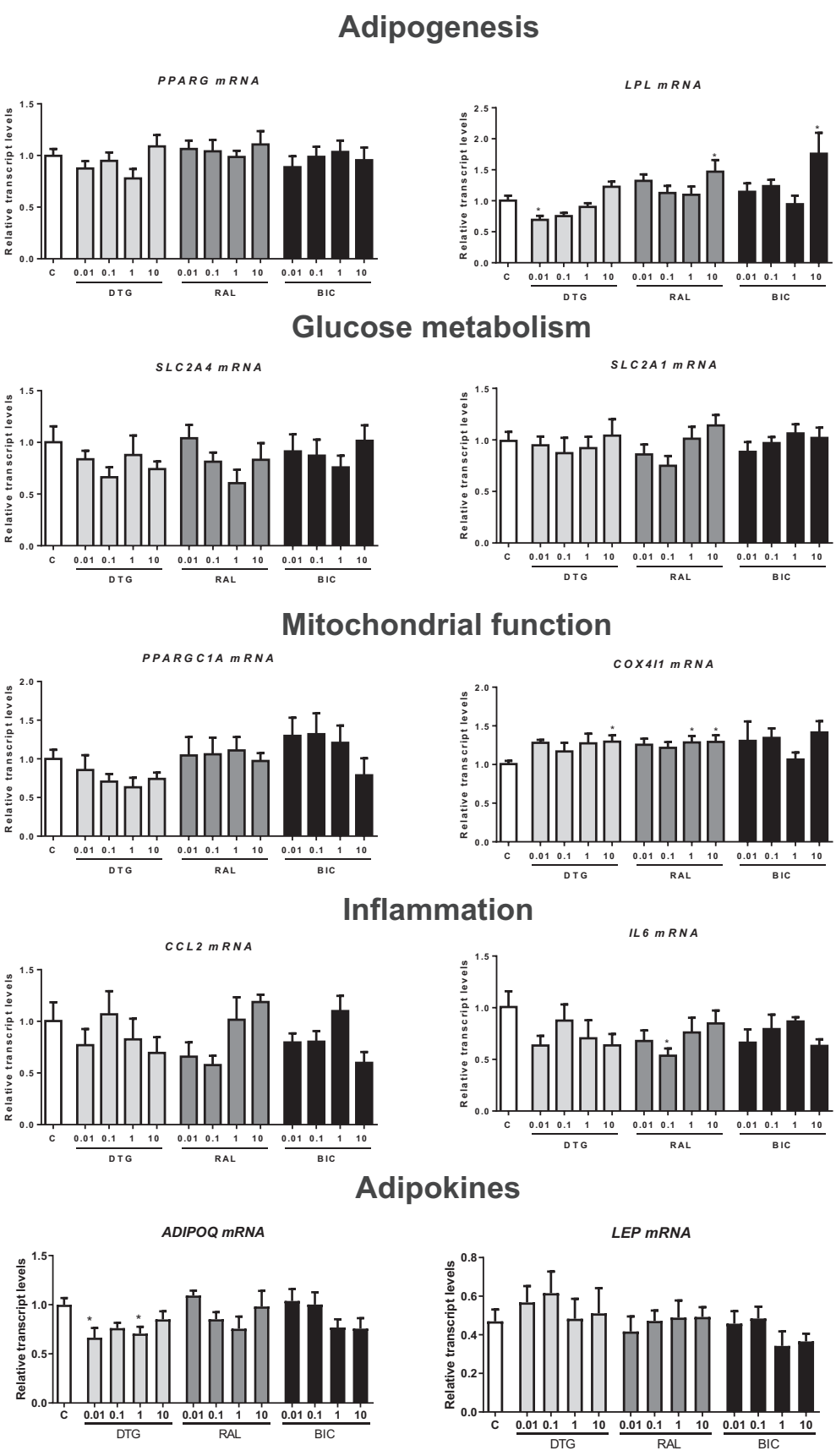

Fig. 4. Effects of dolutegravir (DTG), bictegravir (BIC), and raltegravir (RAL) on the expression levels of genes related to adipogenic function, inflammation, mitochondrial function, and adipokines in differentiated SGBS human adipocytes. SGBS human preadipocytes were differentiated in culture and, on day 14 of differentiation, treated with the indicated concentrations of drugs for 24 h. Data on mRNA levels are presented as means ± SEM from 3 to 4 independent experiments and are expressed relative to values from untreated control cells (defined as 1). *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001 for each drug treatment vs. control.

not reflected in the altered IL6 secretion (Fig. 3).

3.3. Acute effects of INsTIs on human adipocytes

To test the possible acute effects on already differentiated adipocytes, human SGBS pre-adipocytes were cultured and differentiated into adipocytes as previously reported and described in the Materials and Methods section [18]. When the cells had already acquired an adipocyte-like morphology after 14 days of inducing adipogenesis, we examined the effects of INsTIs by exposure for 24 h to DTG, BIC, or RAL (0.01 μM–10 μM). The tested drugs had minimal effects on the assessed gene expression levels (mild induction of LPL expression by RAL and BIC at the highest concentrations) except adiponectin (ADIPOQ). Adiponectin gene expression was significantly decreased by DTG ($P \leq 0.05$) (Fig. 4), although this did not result in a significant change in the levels of adiponectin secreted into the culture medium by the differentiated adipocytes (Supplemental Fig. 1). This may reflect the short duration of drug exposure (24 h) used in the experimental setting.

4. Discussion

Recent evidence suggests an association between anti-retroviral regimens containing INsTIs and increased weight gain in PLWH. This weight gain appeared to be greater in DTG and BIC compared to other INsTIs. The pathophysiological basis of these effects remains unknown.

Consistent with previous reports, this study confirms that RAL does not exert significant effects on adipogenic differentiation or the levels of the tested genes and secreted proteins in human adipocytes [14,22]. DTG did not markedly affect the acquisition of adipocyte morphology (lipid accumulation), which is also in agreement with a recent report using omental and subcutaneous pre-adipocytes [23]. However, we discovered that DTG (but not BIC) at the higher tested concentrations exerted some inhibitory effects on adipocyte gene expression (such as PPARγ and LPL) and markedly reduced adiponectin gene expression and secretion in differentiating adipocytes. Gorwood et al. [24] also reported a decrease in the expression and release of adiponectin and leptin in response to chronic treatment of adipose stem cells (ASCs) with DTG. Meanwhile, and valuable in the translatability of these results to patients, González-Cordón et al. [25] have reported hypoadiponectinemia in PLWH treated with anti-retroviral patterns that include DTG [25].

The downregulation of adiponectin in treated patients might be secondary to increased adiposity [26]. However, our observation that DTG reduces adiponectin in a cell-autonomous adipocyte system suggests a potential direct effect of DTG-based regimens in eliciting hypoadiponectinemia and subsequent deleterious systemic consequences (such as insulin resistance and cardiovascular risk). Notably, we found that adiponectin was not significantly altered in adipocytes treated with BIC, regardless of the differentiation status or concentration, within the tested range. Although some studies have suggested that both DTG- and BIC-containing anti-retroviral regimens favor weight gain [3], our data suggest that DTG has a stronger action in disturbing the adiponectin system in adipocytes. Interestingly, DTG and BIC exhibited opposite effects (induction and repression, respectively) on the expression and release of the pro-inflammatory cytokine IL6, and partially on the expression and release of CCL2 (MCP-1). As adiponectin represses the expression of IL6 in adipocytes [27,28], DTG and BIC may elicit alterations based on the crosstalk between adiponectin and inflammation in adipose tissue.

Finally, we found a mild trend for DTG to decrease leptin expression and release. A recent report showed that leptin levels decreased when patients were switched from protease inhibitor-based to INsTI-containing anti-retroviral regimes [29]. Moreover, given that the use of tenofovir alafenamide (TAF) has also been associated with weight gain in PLWH, the combined and synergistic effects of INsTIs and/or TAF on human adipocytes require further investigation.

This study has some limitations associated with its limited capacity to translate results obtained in vitro into clinical practice. Moreover, besides adipocytes, INsTIs may affect other cell types that are also present in adipose tissue depots. For example, it has been reported that DTG reduces inflammation and senescence in endothelial cells, whereas RAL has no effect [30]. However, the significance of the drug concentrations used in cell culture studies, even if in the range of circulating levels in treated patients, is not unequivocal. DTG has been reported to penetrate adipose tissue cells much more strongly than nucleoside/nucleotide reverse transcriptase inhibitors [31]. Thus, we speculated that the exposure of fat to DTG may be particularly intense in treated patients. Comparable information on intra-adipose concentrations of RAL and BIC is lacking. Despite these limitations, our in vitro study allowed us to establish the direct effects of INsTIs on human adipocytes, which could not be distinctly assessed in studies in patients.

5. Conclusions

In conclusion, our findings show that INsTIs currently used to treat PLWH have differential effects on adipose cells and highlight the potential of DTG to repress adiponectin expression/release and enhance inflammatory cytokine expression/release. Whereas BIC inhibits pro-inflammatory cytokines expression and release. These observations may contribute to improving the treatment profiles used in PLWH to minimize side effects associated with increased adiposity and metabolic alterations.

CRediT authorship contribution statement

Conceptualization, P.D. and M.G.; methodology, T.Q-L., J.V., M.G., F.V.; experimental procedures and analysis, T.Q-L., J.V., M.C., M.M.G., M.G.C., I.M., N.C., J.C.D.; writing—original draft preparation, T.Q-L., M. G., F.V.; review and editing, P.D., T.Q-L, F.V., M.G. All authors have read and agreed to the published version of the manuscript.

Declaration of competing interest

Dr. Pere Domingo reported receiving honoraria from Merck Sharp & Dohme, Gilead Sciences, ViiV Healthcare, Janssen, Cilag, Thera technologies, and Roche. All the other authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this manuscript. The authors guarantee that they have the authority to publish the work and that the manuscript and confirm that neither the manuscript nor any parts of its content are currently under consideration or published in another journal.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2022.120948.

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