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Leisure time physical activity is associated with improved HDL functionality in high cardiovascular risk individuals: a cohort study

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Abbreviations

AMPK: AMP-activated protein kinase

CEC: cholesterol efflux capacity

CETP: cholesteryl ester transfer protein

HDL: high-density lipoprotein

HDL-C: HDL-cholesterol

LTPA: leisure time physical activity

METs: metabolic equivalents of task

PON1: paraoxonase-1

Abstract (250 words)

Aims: Physical activity has consistently been shown to improve cardiovascular health and HDL-cholesterol levels. However, only small and heterogeneous studies have investigated the effect of exercise on HDL functions. Our aim is to evaluate, in the largest observational study to date, the association between leisure time physical activity (LTPA) and a range of HDL functional traits.

Methods: The study sample consisted of 296 Spanish adults at high cardiovascular risk. Usual LTPA and eight measures of HDL functionality were averaged over two measurements, 1 year apart. Multivariable linear regression models were used to explore the association between LTPA (exposure) and each HDL functional trait (outcome), adjusted for cardiovascular risk factors.

Results: Higher levels of LTPA were positively and linearly associated with average levels over 1 year of plasma HDL-cholesterol and apolipoprotein A-I, paraoxonase-1 antioxidant activity, HDL capacity to esterify cholesterol and cholesterol efflux capacity individuals free of type 2 diabetes only. The increased cholesterol esterification index with increasing LTPA reached a plateau at around 300 METS.min/day. In individuals with diabetes, the relationship with cholesteryl ester transfer protein followed a U-shape, with a decreased cholesteryl ester transfer protein activity from 0 to 300 METs.min/day, but increasing from there onwards. Increasing levels of LTPA were associated with poorer HDL vasodilatory capacity.

Conclusions: In a high cardiovascular risk population, LTPA was associated not only with greater circulating levels of HDL-cholesterol, but also with better markers of HDL functionality, namely cholesterol efflux capacity, the capacity of HDL to esterify cholesterol and paraoxonase-1 antioxidant activity in individuals free of diabetes and lower cholesteryl ester transfer protein activity in individuals with type 2 diabetes.

Keywords: HDL function; Physical activity; Lifestyle; Biomarkers

Introduction

Diet and physical activity are key lifestyle factors that can modulate the risk for developing atherosclerotic disease [1]. Epidemiological studies have consistently found that low levels of high-density lipoprotein (HDL) cholesterol (HDL-C) are associated with atherosclerotic cardiovascular disease [2]. Nevertheless, the causal role for HDL-C on CVD risk has been challenged by Mendelian randomization studies [3-5] and unsuccessful pharmacological interventions [6 7]. This has led to the hypothesis that improving HDL function can be more relevant for cardiovascular prevention than raising HDL-C concentrations [8]. Although it is established that exercise increases HDL-C levels [9], its effects on HDL functionality have been less studied and remain to be clarified. As reviewed recently, there is emerging evidence that exercise interventions can improve aspects of HDL functionality in populations at elevated cardiovascular risk (sedentary, overweight/obese, and/or with metabolic syndrome in the majority of studies) [10]. The highest level of evidence was found for cholesterol efflux capacity (CEC), with results from rigorously controlled, large exercise training interventions showing that regular prolonged vigorous exercise improves CEC. For example, in a study of adults with metabolic syndrome weight loss and exercise during 3 months was associated with a 25% increase in CEC [11]. Another study compared different exercise intensities during a 6-month intervention and concluded that CEC only improved with a high dose of high intensity training [12]. However, only a few studies with small samples (median n=33, median duration 3 months) of individuals with metabolic disorders have reported beneficial effects of exercise intervention on other HDL functional properties such as anti-inflammatory and antioxidant [10 13]. Moreover, no observational study has described the effects of real-life levels of physical activity in at-risk populations on a comprehensive set of HDL function traits.

Therefore, the aim of this study is to comprehensively assess the relationship between physical activity and a range of markers of HDL characteristics and functionality in a sample of nearly 300 individuals at high risk of cardiovascular disease.

Methods

Study population

The PREDIMED trial (*PREvencion con DIeta MEDiterranea*) is a dietary intervention which consisted in following a traditional Mediterranean diet supplemented with either virgin olive oil or nuts, and the control group followed a low-fat diet [14]. Individuals were free of cardiovascular disease but had either type 2 diabetes or at least three of the following cardiovascular risk factors: current smoking, hypertension, increased low-density lipoprotein cholesterol, decreased high-density lipoprotein cholesterol, overweight/obesity or family history of premature heart disease. The local Research and Ethics Committee approved the study protocol. All participants gave written informed consent. The trial is registered at http://www.controlled-trials.com/ISRCTN35739639.

The population used for this analysis is a random subsample of 296 participants from the PREDIMED trial [14 15], with equal proportions of participants in each arm of the study (control, Mediterranean diet + extra virgin olive oil, Mediterranean diet + nuts) in which an extensive assessment of HDL functions has been performed at baseline and at 1 year of follow-up [15]. The sample size of 296 was calculated to detect a significant difference of 0.025 points in normalized cholesterol efflux capacity values between pre and post intervention values in the seminal paper that investigated the effect of a Mediterranean dietary intervention on HDL functionality [16]. Only 6 out of the 11 recruiting centres of the PREDIMED trial could provide blood samples for the determination of HDL functionality. Frozen blood samples were shipped to Hospital del Mar Research Institute (IMIM), Barcelona, where all the HDL function assays were performed.

Outcome assessment: HDL function

Detailed description of HDL function assays, including coefficients of variation and detailed protocols, has been published elsewhere [16 17]. Participants' HDL cholesterol and apolipoprotein

A-I (ApoA-I) levels were analysed in an ABX-Pentra 400 autoanalyzer (Horiba ABX, Montpellier, France). HDL particles were first isolated from plasma samples by density gradient ultracentrifugation (isolated HDL fraction) or polyethylene glycol-induced precipitation of apolipoprotein B-containing lipoproteins (apolipoprotein B depleted plasma), and the samples were stored at –80 °C until use. The following properties were determined as previously described [16 17]: 1) CEC in a model of human THP-1 monocyte-derived macrophages with 3H-cholesterol treated with 5% apolipoprotein B-depleted plasma samples; 2) the ability of HDLs to esterify cholesterol (HDL esterification index) as the ratio between the percentage of esterified cholesterol in isolated HDL plasma samples and lecithin cholesterol acyltransferase concentration in serum samples; 3) HDL inflammatory index, an indirect measurement of the ability of apolipoprotein B-depleted particles to protect low-density lipoprotein from oxidation, based on the oxidation of the fluorescent 2',7'-dichlorodihydrofluorescein; 4) the activities of cholesteryl ester transfer protein (CETP) and paraoxonase-1 (PON1) enzymes in plasma and serum samples, respectively, by commercial kits; 5) HDL vasodilatory capacity as the capacity to promote endothelial release of nitric oxide in vitro in a human umbilical vein endothelial cell model treated with apolipoprotein B-depleted plasma samples. Due to sample availability and technical issues, there were 67 missing values for CETP and PON1 activity, and 50 missing for HDL vasodilatory capacity. Cumulative average of the baseline and 1-year follow up measure was calculated and used as primary outcome variable, to increase precision [18].

Exposure assessment: Physical activity

Non-occupational physical activity was assessed at baseline and at 1 year by the self-administered Minnesota Leisure Time Physical Activity (LTPA) Questionnaire. The Spanish version, consisting of 67 activities organized in nine sections, has been validated in men and women [19 20]. Participants were asked to indicate the number of days and minutes per day they had performed those activities during the previous year. Physical activity was quantified in metabolic equivalents of task in minutes per day (METs.min/day) by multiplying the METs of

each activity with its mean duration (in minutes per day). LTPA was classified as light LTPA (intensity <4 METs, e.g. walking), moderate LTPA (4–5.5 METs, e.g. brisk walking) and vigorous (≥6 METs, e.g. jogging). Similar to the outcomes, cumulative average of the baseline and 1-year follow up measure was calculated and used as primary exposure variable, to better reflect usual physical activity.

Covariates

Clinical variables were measured by a trained nurse (weight, height, blood pressure, and biochemical profile). Questionnaires were used to collect information on medication, smoking habits, and adherence to a traditional Mediterranean diet by a validated 14-item screener [21]. Body mass index (BMI) was calculated as the ratio between weight (kg) and the height squared (m^2). BMI and Mediterranean diet score were averaged over the baseline and 1-year values. Dyslipidaemia was defined by presence of any of the following: hypercholesterolemia defined as total cholesterol ≥200 mg dL^{-1} or the use of statins; hypertriglyceridemia defined as triglycerides ≥150 mg dL^{-1} and/or the use of fibrates or pharmacological doses of omega-3 polyunsaturated fatty acids. Type 2 diabetes mellitus was defined as the presence of an altered glucose metabolism or the use of antidiabetic drugs. Hypertension was defined as systolic blood pressure ≥140 mmHg, diastolic blood pressure ≥90 mmHg, or the use of antihypertensive agents.

Statistical analysis

Quintiles of cumulative average of physical activity were created. We describe the sample characteristics overall across quintiles of LTPA and calculated the p-value for linear trend across quintiles using contrasts. For the main analysis, the outcomes of interest were each HDL function trait (cumulative average), standardized to z-scores with a mean of 0 and a standard deviation of 1, so that all estimates can be comparable. We used separate generalized linear models for each HDL function trait as dependent variables, and different levels of LTPA as independent variables, treated as continuous. The beta coefficients from these regressions are

given for an increase in 100 METs.min/day. This corresponds for example to 25 min of brisk walking (a moderate physical activity, estimated at 4 METs) per day, thus 175 min per week. The main results are given for LTPA as the sum of light, moderate and vigorous LTPA. Diabetes being a strong correlate of HDL functionality [22], we tested for interaction by introducing a cross-product term between LTPA and diabetes status, and stratified the analyses by type 2 diabetes status. To investigate the shape of the association, LTPA variables were also modelled in quintiles and predicted adjusted means across quintiles are presented. All models were adjusted for sex, intervention group, study centre, and baseline age, body mass index, smoking status, score of adherence to a traditional Mediterranean diet, dyslipidaemia, type 2 diabetes and hypertension status. To better assess the potential non-linear dose-response relationship, restricted cubic splines were fitted. Likelihood ratio tests between fully adjusted nested models, the first with only the linear term, and the second with the restricted cubic spline terms were performed. The resulting p-value is denoted as "p-value non-linear". To avoid distortion of the spline curves by extreme values, we excluded LTPA values >1000 METs.min/day (n=4) for the spline analysis. All analyses were conducted using Stata 14.0 (Stata Corp) and the "rcs" package in R Software, version 3.5.0. All tests were two-sided with an alpha level of 0.05.

Results

Participants' characteristics are described in Table 1. Median LTPA was 201 METs.min/day, coming mostly from activities of low intensity (115 METs.min/day). Expressed in METs.min/week, this is 1407 METs.min/week, which is more than twice the minimum recommended by the World Health Organization of 600 METs.min/week of total LTPA. The levels of LTPA in the first quintiles (2.0-86.5 METs.min/day) are below the recommended minimum amount. The majority of characteristics did not show a statistically significant trend across quintiles of LTPA, with the exception of a lower proportion of women $p < 0.001$.

As seen in Figure 1 by quintile and Figure 2 in the spline analysis, higher levels of LTPA were positively and linearly associated with HDL-C concentrations after adjustment for confounders, despite not reaching conventional statistical significance ($\beta_{100METs}$=0.058 (95% CI: -0.007, 0.123), p=0.081). Unsurprisingly as it is the major protein constituent of HDL-C, plasma ApoA-I levels also tended to increase as LTPA levels do, although reaching a plateau after 400 METs.min/day, which corresponds to the fifth quintile. Regarding HDL functionality, there was evidence of a positive association of LTPA with PON1 antioxidant activity ($\beta_{100METs}$=0.070 (95% CI: -0.008, 0.148), p=0.079). The relationship with HDL capacity to esterify cholesterol was not linear (p-value non-linear=0.078), and a model using restricted cubic splines with 3 knots shows that the increase observed in cholesterol esterification index from 0 to 300 METs.min/d plateaus from 300 METs.min/d onwards (Figure 2). The relationship with CETP was also non-linear, as the model with cubic splines had a significantly better fit (p=0.022), and reveals a U-shape, with decreasing CETP activity as LTPA increase from 0 to 300 METs.min/day, where it then plateaus until 400 METs.min/d, beyond which CETP activity increases as LTPA increases (Figure 2). Surprisingly, increasing levels of LTPA were associated with lower HDL vasodilatory capacity (Figure 1, $\beta_{100METs}$=-0.058 (95% CI: -0.115, -0.001), p=0.049). However, the cubic spline analysis reveals that a model with 7 knots has a significantly better fit than the linear model (p=0.020), showing an oscillating relationship (Figure 2). There was no clear evidence of any association between LTPA and cholesterol efflux capacity, nor with HDL inflammatory index. The splines show that, for the HDL functional traits that display an association with physical activity, compared with no physical activity (0 METs.min/day), the effects are significant from approximately 80-100 METs.min/day (where the confidence interval crosses the x-axis), which corresponds to the minimum amount of physical activity recommended by the World Health Organization [23].

Characteristics of participants according to diabetes status are presented in Supplemental Table 1. When stratifying by diabetes status (Figures 3 and 4), all associations, except for CETP, were only apparent in non-diabetic individuals. In this group, we observe that

LTPA was strongly and linearly associated with HDL-cholesterol, apolipoprotein A-I, paraoxonase-1 antioxidant activity as well as cholesterol efflux capacity, and with HDL capacity to esterify cholesterol with a plateau at 300 METs.min/day. The associations with these traits were inexistent in diabetic individuals. Conversely, LTPA displayed a U-shape association with CETP activity in the diabetes group, but not in non-diabetic individuals. The inverse association with vasodilatory index was also more apparent in diabetic than in non-diabetic people.

Discussion

In a sample of 296 individuals at high cardiovascular risk, we found that LTPA was associated not only with greater circulating levels of HDL-C and ApoA-I, but also with better markers of HDL functionality, namely greater CEC, capacity of HDL to esterify cholesterol and PON1 antioxidant activity in non-diabetic subjects, and lower CETP activity in individuals with diabetes.

Physical activity has been shown to increase HDL-C levels in a large number of human trials and observational studies[8], even in subjects at elevated risk of cardiovascular disease [24]. Our results support this association, which has been traditionally explained by the capacity of aerobic exercise to promote the activation of AMP-activated protein kinase (AMPK)[25]. AMPK is a key element involved in the regulation of cell metabolism at states of low metabolic energy[26]. Its activation seems to be partially responsible for the benefits of physical activity on lipid metabolism, glucose homeostasis, antioxidant/anti-inflammatory protection and other pathways involved in the development of chronic diseases[27]. Regarding HDL-related specific actions, AMPK is able to stimulate the peroxisome proliferator-activated receptor alpha, a transcription factor capable of promoting the hepatic synthesis of ApoA-I, leading to increases

in HDL-C circulating levels [28]. Moreover, its stimulation could also be partially responsible for the promotion of HDL anti-atherosclerotic functions.

Beyond the increments in the quantity of cholesterol circulating in HDL particles, we observed in the present study an association between LTPA and greater values of CEC, HDL ability to esterify cholesterol, and the activity of the HDL-bound antioxidant enzyme PON1. Despite not directly comparable due to design differences, the observed results of CEC are in line with outcomes of exercise interventions, lasting between 3 and 12 months in CVD patients or individuals at elevated CVD risk, that showed overall improvements in CEC up to a 25% increase [10]. Shorter interventions (median of 3 months) also indicate some antioxidant effect of exercise on the ability of HDL to transport lipid peroxides [10 13]. Antioxidant properties of AMPK make biologically plausible the association of LTPA with these traits. On the one hand, AMPK is known to be able to promote the phosphorylation of forkhead box protein O1, a transcription factor capable of upregulating the expression of antioxidant enzymes such as superoxide dismutase and catalase [29 30]. On the other hand, AMPK is also able to induce the activation of the nuclear factor erythroid 2-related factor 2, another transcriptional regulator capable of promoting the expression of antioxidant enzymes such as NAD(P)H dehydrogenase or glutathione S-transferase, among others [31]. The combination of the previous two mechanisms may result in less oxidative modifications of HDL proteins, such as ApoA-I, lecithin cholesterol acyltransferase, and PON1. These three proteins play a pivotal role in CEC, HDL capacity to esterify cholesterol, and antioxidant defences, and are known to partially lose their function when they become oxidized [32-34]. Therefore, an AMPK-mediated antioxidant protection could contribute to explaining the improved HDL functions in individuals with greater levels of LTPA. There was no clear association with HDL inflammatory index. Intervention studies are scant and have failed to produce consistent results on the effect of exercise on HDL anti-inflammatory capacity [10 13]. A recent study in patients with hypertension found an effect of an aerobic intervention on antioxidant but not anti-inflammatory capacity of HDL, measured by cell-free assays [13].

HDL is more dysfunctional in individuals with type 2 diabetes [22], therefore to rule out the confounding effect of diabetes, we stratified our results and found that most associations were only apparent in non-diabetic people. The interpretation of these results is that there is more scope for improvement of HDL function by increasing physical activity levels in non-diabetic patients, whereas in type 2 diabetes patients, the functionality of HDL shows little difference according to physical activity. This is in line with a recent review of the evidence that finds only limited effect of exercise training, in particular of resistance training, on lipid parameters in type 2 diabetes patients [35]. The exception was for CETP activity, that displayed a U-shape relationship with LTPA only in diabetics. We observed a decrease in the CETP activity with increasing physical activity up to moderate/high levels (300 METs·min/d), which correspond to approximately one hour of brisk walking or 30 min of jogging per day, every day. Considering that this enzyme requires a source of triglycerides to exchange for cholesteryl esters from HDL particles [36], and that circulating triglyceride levels are greater in diabetics patients, a decrease in triglycerides in circulation, related to the physical activity practice, may be partially responsible for a slight moderation in its function [24].

Finally, we observed that HDL vasodilatory capacity, measured by the release of nitric oxide, seemed to decrease when levels of LTPA increase, although the relationship was unclear and showed oscillations in the spline analysis that are not interpretable. The majority of studies that have studied the effect of exercise on nitric oxide have investigated its immediate acute effect on nitric oxide release in exhaled air. These studies show either increased, decreased or unchanged levels of exhaled nitric oxide in response to exercise, probably depending on factors such as the levels of nitric oxide synthase, the severity of oxidative stress, nitric oxide binding to antioxidant molecules, and also individual patterns of physical activity [37]. Moreover, long-term exposure to physical activity is not comparable with short-term changes, and the association observed here may also be mediated by potential changes in some key HDL components, such as acute phase HDL proteins. Due to the ambivalent nature of nitric oxide in cardiovascular health, further research regarding the association between long-term higher levels of physical activity and HDL vasodilatory capacity values is necessary.

Taking physical inactivity (0 METs.min/day in LTPA) as the reference, most associations were observed from 100 METs.min/day (700 METs.min/week), which corresponds to 25 min of brisk walking per day, and is just above the World Health Organization guidelines recommendation [23]. This is consistent with results from a recent meta-analysis of prospective studies that showed a decrease in the risk of a wide range of chronic disease, including cardiovascular disease and cancer from levels of 86 METs.min/day (600 METs.min/week) [38]. Moreover, our data suggest no additional benefits beyond 300 METs.min/day (2100 METs.min/week), which is consistent with analyses linking physical activity with the risk of ischaemic heart disease and stroke that show that major gains were achieved at lower levels of physical activity, whereas the decrease in risk flattens and becomes minimal at higher levels than 3000-4000 METs.min/week [38].

Strengths and limitations

Our study has several strengths, including the novelty of investigating physical activity in an observational setting in relation to HDL functionality, the prospective cumulative average assessment of physical activity and HDL functionality to increase precision, the comprehensive assessment of an array of HDL-related functional traits using standardized protocols, and the relatively large sample size compared to studies in the HDL functionality literature. However, several limitations should be considered when interpreting the present findings. First, the population under study consisted of older adults at high risk of cardiovascular disease, who present characteristics, including more dysfunctional HDL, that cannot be generalizable to healthier populations. Second, this study was a dietary intervention trial which did not include any physical activity advice. As a consequence, we analysed it as a cohort study, and tried to overcome this limitation by adjusting for the intervention group and by taking as the exposure the cumulative average of physical activity from baseline and at 1-year, which takes into account any change in physical activity during the first year of the trial. Third, the levels of physical activity were self-reported, and not objectively measured, therefore prone to measurement error. Fourth, we used cellular models to determine two HDL functions (CEC and vasodilatory capacity), which may not reflect the biological interplay and potential counter-

regulatory mechanisms occurring. Fifth, the capacity of HDL to esterify cholesterol determination was done on HDL isolated by ultracentrifugation, whereas the other assays were performed on apolipoprotein-B depleted plasma, which might have yielded different amount of isolated HDLs. Finally, although the total sample size was nearly 300 participants, there were substantial missing data on three assays, namely 23% for CETP and PON1 activity and 17% for vasodilatory capacity. However, it is reasonable to assume that these data were missing completely at random, and do not affect the representativity of the subsample, only larger standard errors and loss of statistical power.

To conclude, we present the first study to assess the long-term associations of real-life physical activity levels and a comprehensive set of HDL functionality properties in high cardiovascular risk older individuals. We find that not only is leisure time physical activity associated with greater levels of HDL-C and ApoA-I, but also with more functional HDL, with greater CEC, esterification capacity, PON1 activity in individuals free of diabetes, and lower CETP activity at intermediate physical activity levels in type 2 diabetes patients. Considering the functionality of HDL rather than its cholesterol content is increasingly gaining interest as a predictor of risk of CVD, therefore more longitudinal studies are needed to evaluate how physical activity influences HDL-related functional traits.

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Conflict of interest

X.P. reports personal fees from Abbott, Esteve, Lacer, Rubio, and Sanofi outside the submitted work. R.E. reports grants from Bicentury SA, Cerveza y Salud, Grand Fountaine, and Novartis SA; and personal fees from Brewers of Europe, FIVIN, Fundación Cerveza y Salud, Lilly Laboratories, and Wine and Culinary International Forum outside the submitted work. J.S.-S. reports to be a board member and grants from Nut and Dried Fruit; personal fees from Aguas Font Vella Lanjarón, Danone S.A., and Instituto Danone; grants from Eroski Distributors; and non-financial support from Nuts for Life outside the submitted work. E.R. reports personal fees and non-financial support from Merck, Sharp & Dohme; grants, personal fees, and non-financial support from California Walnut Commission; grants and personal fees from Sanofi; personal fees and non-financial support from Ferrer International; and grants from Pfizer outside the submitted work. The rest of the authors have nothing to disclose.

Authors' contributions

C.L. designed and conducted the statistical analysis. A.H. and M.Fitó designed the study and A.H. acquired the data. M.A.M.-G., E.R., X.P., R.E., J.S.-S., D.C., A.A.G., L.S.-M., M.Fiol, J.L., E.G.-G., and M.Fitó contributed with biological samples and/or participated in the design and development of the clinical trial. C.L and A.H. drafted the manuscript, which was critically revised by M.T.S.-F., O.C., H.S., M.A.M.-G., E.R., X.P., R.E., J.S.-S., D.C., A.A.G., L.S.-M., M.Fiol, J.L., E.G.-G., and M.Fitó.

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Table 1. Baseline participants characteristics overall and across quintiles of cumulative average of leisure time physical activity, subsample of the PREDIMED study

	Leisure time physical activity total							
	All	Q1 (2.0-86.5)[1]	Q2 (87.2-159.1)[1]	Q3 (159.5-251.1)[1]	Q4 (252.2-383.5)[1]	Q5 (385.0-2188.1)[1]	p-value	N
	N=296	N=60	N=59	N=59	N=59	N=59		
Age (y)	65.9 (6.43)	66.4 (7.10)	65.3 (7.00)	65.2 (6.57)	67.4 (5.86)	65.4 (5.36)	0.293	296
Female sex (%)	151 (51.0%)	41 (68.3%)	35 (59.3%)	32 (54.2%)	26 (44.1%)	17 (28.8%)	<0.001	296
Smoking (%)	37 (12.5%)	8 (13.3%)	8 (13.6%)	8 (13.6%)	6 (10.2%)	7 (11.9%)	0.976	296
Type-2 diabetes mellitus (%)	123 (41.6%)	25 (41.7%)	20 (33.9%)	24 (40.7%)	22 (37.3%)	32 (54.2%)	0.215	296
Hypertension (%)	259 (87.5%)	52 (86.7%)	54 (91.5%)	52 (88.1%)	52 (88.1%)	49 (83.1%)	0.731	296
Dyslipidemia (%)	236 (79.7%)	49 (81.7%)	42 (71.2%)	44 (74.6%)	50 (84.7%)	51 (86.4%)	0.175	296
Mediterranean diet score (0-14)[2]	9.37 (1.43)	9.08 (1.48)	9.34 (1.41)	9.50 (1.23)	9.41 (1.50)	9.50 (1.54)	0.488	295
BMI (kg/m2)[2]	29.6 (3.83)	30.2 (3.94)	30.0 (4.13)	29.6 (3.77)	29.1 (3.40)	29.2 (3.88)	0.483	296
Exposure[2,3]								
Total LTPA (METs·min/day)	201.3 (101.3 - 330.8)	44.6 (17.5 - 69.5)	117.5 (102.1 - 132)	201.6 (175.1 - 220.1)	301.1 (281 - 331.5)	490.1 (425.1 - 690.6)	<0.001	296
Light PA	87.5 (21.4 - 170.35)	18.8 (3.8 - 44.6)	62 (21.5 - 98.5)	107 (58 - 171.6)	166.6 (113.5 - 233)	182 (49.3 - 315)	<0.001	296
Moderate to vigorous PA	76.3 (13.5 - 180.54)	2 (0 - 31.3)	53.5 (0 - 87.5)	78 (36 - 145.5)	148 (56.5 - 195)	371.5 (191.6 - 556.5)	<0.001	296
Outcome[2]								
HDL-cholesterol (mg/dL)	49.3 (10.9)	50.5 (10.0)	49.8 (11.9)	47.3 (10.4)	50.2 (11.9)	48.7 (10.3)	0.514	288
Plasma Apolipoprotein A-I (mg/dL)	135 (20.0)	133 (18.3)	133 (20.7)	135 (22.4)	138 (19.1)	136 (19.6)	0.841	219
Cholesterol efflux capacity[4]	0.95 (0.09)	0.96 (0.09)	0.93 (0.10)	0.93 (0.10)	0.97 (0.10)	0.95 (0.09)	0.278	264
HDL-C esterification index[4]	6.43 (1.55)	5.69 (1.80)	6.69 (1.60)	6.92 (1.28)	6.26 (1.45)	6.64 (1.36)	0.006	180
Cholesteryl ester transfer protein activity[4]	1.11 (0.18)	1.15 (0.19)	1.13 (0.19)	1.07 (0.15)	1.09 (0.17)	1.09 (0.17)	0.321	184
Paraoxonase-1 antioxidant activity[4]	0.90 (0.36)	0.83 (0.38)	0.92 (0.39)	0.95 (0.39)	0.84 (0.30)	0.94 (0.35)	0.484	182
HDL inflammatory index[4]	0.97 (0.15)	0.97 (0.15)	0.94 (0.18)	0.97 (0.14)	0.97 (0.13)	0.99 (0.16)	0.561	282
HDL vasodilatory capacity[5]	1.13 (0.28)	1.20 (0.34)	1.10 (0.20)	1.17 (0.31)	1.10 (0.24)	1.11 (0.28)	0.26	237

[1] minimum and maximum value of LTPA in the quintile
[2] Cumulative average between baseline and 1 year
[3] Median (interquartile range)
[4] normalized unit
[5] unitless ratio

Figure captions

Figure 1. Multivariable† estimated standardized mean z-score values and 95% confidence intervals of HDL-related traits across quintiles of leisure time physical activity and multivariable regression coefficients from generalized linear model regressions

† Estimates are adjusted mean z-score and 95% confidence intervals in each quintile of LTPA, adjusted for sex, intervention group, study centre, and baseline age, body mass index, smoking status, Mediterranean diet score, dyslipidaemia, type 2 diabetes and hypertension status

Significance of the comparison with the first quintile is given by the asterisks on the graph * $p<0.05$, ** $p<0.01$

Figure 2. Dose-response association† of HDL-related functional traits assessed by restricted cubic splines

† Adjusted for sex, intervention group, study centre, and baseline age, body mass index, smoking status, Mediterranean diet score, dyslipidaemia, type 2 diabetes and hypertension status

Figure 3. Multivariable† estimated standardized values and 95% confidence intervals of HDL-related traits across quintiles of leisure time physical activity and multivariable regression coefficients from generalized linear model regressions by diabetes status

† Estimates are adjusted for sex, intervention group, study centre, and baseline age, body mass index, smoking status, Mediterranean diet score, dyslipidaemia, and hypertension status

Significance of the comparison with the first quintile is given by the asterisks on the graph * $p<0.05$, ** $p<0.01$

p-value for interaction between LTPA and diabetes: HDL-cholesterol p=0.305, Apolipoprotein A-I p=0.673, Cholesterol efflux capacity p=0.242, HDL-C esterification index p=0.100, Cholesteryl ester transfer protein activity p=0.078, Paraoxonase-1 antioxidant activity p=0.054, HDL inflammatory index p=0.152, HDL vasodilatory capacity p=0.719.

Figure 4. Dose-response association† of HDL-related functional traits assessed by restricted cubic splines, by diabetes status

† Adjusted for sex, intervention group, study centre, and baseline age, body mass index, smoking status, Mediterranean diet score, dyslipidaemia, and hypertension status

Supplemental Table 1. Baseline participants characteristics by Type 2 Diabetes status, subsample of the PREDIMED study

	Type 2 diabetes status		
	No	Yes	p-value
	N=173	N=123	
Age (y)	65.3 (5.99)	66.8 (6.94)	0.064
Female sex (%)	101 (58.4%)	50 (40.7%)	0.004
Smoking (%)	25 (14.5%)	12 (9.76%)	0.305
Hypertension (%)	152 (87.9%)	107 (87.0%)	0.964
Dyslipidemia (%)	140 (80.9%)	96 (78.0%)	0.646
Mediterranean diet score (0-14) [1]	9.47 (1.33)	9.22 (1.56)	0.16
BMI (kg/m2) [1]	29.5 (3.86)	29.8 (3.79)	0.572
Total LTPA (METs·min/day) [1,2]	179 (100 - 301)	212 (97 - 348)	0.024
Light PA[1,2]	83 (18 - 159)	92 (21 - 176)	0.12
Moderate to vigorous PA[1,2]	77 (16 - 158)	67 (7 - 186)	0.067
Triglycerides (mg/dL)	119 (59.4)	140 (121)	0.084
HDL-cholesterol (mg/dL) [1]	51.9 (11.0)	45.8 (9.77)	<0.001
Plasma Apolipoprotein A-I (mg/dL) [1]	138 (19.3)	131 (20.4)	0.019
Cholesterol efflux capacity [1,3]	0.96 (0.09)	0.93 (0.09)	0.003
HDL-C esterification index [1,3]	6.37 (1.49)	6.52 (1.64)	0.549
Cholesteryl ester transfer protein activity [1,3]	1.12 (0.18)	1.09 (0.17)	0.175
Paraoxonase-1 antioxidant activity [1,3]	0.93 (0.41)	0.85 (0.29)	0.161
HDL inflammatory index [1,3]	0.97 (0.16)	0.97 (0.15)	0.894
HDL vasodilatory capacity [1,4]	1.11 (0.27)	1.16 (0.29)	0.21

[1] Cumulative average between baseline and 1 year
[2] Median (interquartile range)
[3] normalized unit
[4] unitless ratio