Double Blinded Clinical Study on D-Glyceric Acid, a Potential Novel Energy Metabolic Signaling Metabolite
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ABSTRACT

Based on earlier few studies natural metabolite D-glyceric acid does not seem to play any major role in cellular metabolism. Nevertheless, one study that concentrated on ethanol oxidation in the liver increased our curiosity. Therefore, research leading to the present 25 days lasting double blinded human study with placebo control was initiated. Main targets in the present study were: 1) to find out whether acute and long-term exogenous D-glyceric acid (DGA) regimen will cause activation related to energy metabolism in healthy 50-60-year old humans, and 2) to find out whether elevated levels of endogenous DGA can be found in the plasma after 30 min of a strenuous cycling exercise. Additional target was to find out whether certain effects of exogenous DGA regimen resemble the effects of physical exercises, and whether lowered halved dose of DGA regimen during a 14-day follow-up period continues to be effective.

The main results revealed that: I) there was an acute effect on plasma glucose maintenance from DGA regimen, II) plasma metabolites and gene expressions related to aerobic energy production were strongly modulated, and subclinical inflammation was lowered already after the 4-day DGA regimen, and III) cycling with increasing load towards exhaustion increased endogenous levels of DGA in human plasma (p=0.013).

Because the amounts of administered DGA were exceedingly small and cannot directly have caused observed substantial changes, we hypothesise that an increase in tissue DGA concentration causes an endogenous signal or a cascade of intracellular signals: “more ATP is needed immediately”. At the whole body level, the signal causes metabolic activation cascade that resembles initiation of physical exercises but without literally no signs of exhaustion. Tested exogenous DGA regimen possessed an effect even on circulating immune cells. Based on obtained results, the DGA regimen may facilitate in resolving energy metabolic challenges in main organs, like fatty liver, and simultaneously it seemed to lower systemic inflammation. The DGA regimen can possibly alleviate chronic diseases and disorders related to energy metabolism and/or elevated subclinical inflammation.

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Keywords: energy metabolism (EM), OXPHOS, DGA-signal, replenishing
Introduction

D-glyceraldehyde (DGA) is a natural organic acid present in vertebrates and plants, but however, there are only few scientific studies on this small metabolite. Due to its small size and low varying concentrations even the measurement of exact DGA concentration from fluids and tissues at physiological levels is somewhat challenging [1]. In 27-39 year-old adults, the DGA concentration in plasma was on average only 0.3 % of the lactate concentration [2]. As a small monocarboxylic acid, DGA molecule distributes easily through monocarboxylate transporters from blood circulation into tissues. Interestingly in pediatric patients, the ratio of DGA concentration in cerebrospinal fluid to plasma seems to be clearly higher than in organic acids in general [2]. This may indicate that the diffusion of DGA through blood brain barrier and also other membranes is not totally fluent.

Main enzyme that metabolizes DGA in humans and animals is glycerate kinase (GLYCTK). Also, glyoxylate reductase hydroxypyruvate reductase (GRHPR) can oxidize DGA. GLYCTK and GRHPR are widely expressed and active in all tissues (www.proteinatlas.org/). In some tissues DGA can also be reduced through aldehyde oxidase (AOX1 enzyme). GLYCTK enzymes possess several splice variants that are localized into cytosol and mitochondria [3]. According to Uniprot Knowledgebase, 4 splice variants are located into mitochondria and 3 into cytosol. GLYCTK kinase reaction consumes one ATP and simultaneously phosphorylates DGA into glycolytic intermediate, 2-phosphoglycerate. There is a rare inborn error called D-glyceraldehyde Aciduria that likely relates to the deficiency in GLYCTK [4].

Cellular synthesis of DGA occurs mainly from D-glyceraldehyde (D-GALD) via aldehyde dehydrogenase (ALDH) enzymes. These enzymes are often mitochondrialy located [5]. D-GALD is a product of fructose catabolism that occurs mainly in the liver and to a lesser extend in intestines. Interestingly, skeletal muscles and many other organs also possess GLUT transporters that are specified into fructose intake from plasma indicating clearly that D-GALD producing fructose catabolism occurs to some extent also in skeletal muscle [6, 7]. In normal physiological conditions D-GALD is phosphorylated in the cytosol by triose kinase and it enters glycolysis, not towards DGA. Additionally, cellular synthesis of DGA may occur also from hydroxypyruvate (HPA) that is reduced to DGA by cytosolic GRHPR enzyme. Hepatic and renal tissues possess peroxisomal and mitochondrial aminotransferase enzymes (AGXTs) that provide HPA and an amino acid.

In earlier studies the effects of DGA administration have been linked to ethanol [8, 9]. Oxidation of ethanol was shown to accelerate by substantial 25% in male rats during 1 and 2 hours after 1.2 g/kg intraperitoneal ethanol dose, when acute 500 mg/kg or 100 mg/kg DGA dose was given intraperitoneally one hour before ethanol dose [8]. Due to energy metabolic homeostasis ethanol oxidation in the liver occurs typically at a very constant rate and thus this temporary acceleration was very remarkable. Eriksson et al. proposed specific stoichiometric mechanisms of action (MoA) to these remarkable findings but did not verify them with flux calculations. Habe et al. [9] studied gastric cells in vitro with 2 – 3 % ethanol-dosed medium. In their experiments cell viability increased in 72 h by certain doses of DGA, LGA or racemic DL-GA. Eriksson et al. implicitly assumed that DGA metabolism occurs mainly in the liver but inter alia Habe’s experiment showed clearly that DGA is actively metabolized also in other tissues [8, 9]. When the assumption of predominantly hepatic metabolism of DGA is abandoned, it can be unambiguously shown by flux calculations that the postulated MoA of DGA in [8] cannot hold.

The need to find out more plausible MoA that could explain the results adequately from all relevant angels led to further in vitro and in vivo research and eventually to the present human study. Fast activation of overall energy metabolism (EM) by the DGA administration was initially chosen as the main objective of our studies because that explanation was clearly the most likely cause of observed 25% enhancement of alcohol oxidation in rats [8] when the assumption that DGA is metabolized only in the liver was abandoned.
In the following, the activation of EM means the activation of energy production and its consumption. EM activation may also include enhancements of other pathways that directly facilitate EM such as phosphagen system, various membrane transporters, shuttling systems etc. Cellular energy is mostly produced from mitochondrial OXPHOS in the form of ATP. High energy NADH molecules provide most of the reducing power for OXPHOS.

The aim of the present study was to find out whether overall cellular EM was activated acutely and in the long-term under the DGA administration in healthy 50-60-year old volunteers, and secondly whether endogenous DGA concentration in plasma rises after a bout of strenuous exercises. Simultaneously as an inevitable follow-up to the main objectives we wanted to find out, whether 1) the acute DGA response resembles the response from a strenuous bout of physical exercise, as well as 2) does long-term DGA regimen possess similar physiological changes than adaptation to more active physical lifestyle. This may lead to positive changes in health risk factors such as a reduction in low-grade inflammation [10, 11] and improvement blood lipid and glucose [12] profiles.

Materials and methods

Altogether 27 apparently healthy 50-60 year-old volunteers were tested (16 females and 11 males). Their average age was 56.0 years (from 50.3 to 60.9 yrs.), BMI 25.3 (20.1-31.7), and maximal oxygen uptake (VO\textsubscript{2max}) 35.5 ml/kg/min (21.8-48.8 ml/kg/min). Age group of 50-60 -years was chosen because at that age systemic inflammation markers are on average somewhat elevated even in apparently healthy persons. Body mass index (BMI) was restricted to below 32 so that there could be no obese persons among the participants.

Selection Criteria and Monitoring of Studied Persons

Selection criteria for the participants were that they are healthy and belonged to the studied age group and that they could maintain stable living conditions during the whole study. All participants agreed to conduct normal and stable food intake, physical activity, and other daily routines (like work) starting already 5 days before the first 0-control measurement and continue that throughout the whole study period. The weekday of the measurements (Friday or Saturday) was always the same enabling stable conditions. Those persons who had to travel extensively during the study were excluded. Health status of each participant was checked personally with pre-questioner and in the information event before the final selection. Normal and stable behavior was checked by personal diaries and reminder emails, and additionally in morning interviews when arriving to the study site in minimum 30 minutes (min) before the first blood sample. All the participants who came to the Day0 measurements completed the whole study. Nevertheless, two persons had to be excluded from final results because of fully unintended deviations from strictly stable circumstances, one from both the DGA and placebo groups.

Study Setup

The test setup was double-blinded. The placebo group was chosen randomly among females and males separately in beforehand, consisting of 10/27 in the placebo and 17/27 in the DGA group. In the placebo group starting VO\textsubscript{2max} turned out to be 2.9 % better compared to the DGA group. Random placebo selection caused also average age and BMI to deviate 2.2% and 3.6% respectively between the placebo and DGA groups. These small random differences were not even close to be statistically significant.

Three morning fasting and resting blood samples drawn from antecubital vein at Day0, Day7 and Day21 represent “the non-acute measurements” in the study (Fig. 1). In the non-acute measurements, the last dose of the test item or placebo was taken 12 hours before the first blood sample. Sufficient size of the last dose 12 hours before measurement days (Day7 and Day21) was emphasized to participants and checked next morning in the coming interview. In order to avoid possible effects from the strenuous VO\textsubscript{2max} test on
the results, a two day recovery period was added (Fig. 1). Furthermore, by adding the 2 recovery days, all the measurements could be taken at the same weekday. The DGA regimen started only after 2 recovery days and lasted for 4 days before Day7 non-acute measurement (Fig. 1). In the following, we refer to this period also as “the 4-day change” to emphasize that the effects at Day7, if any, were from the 4-day DGA regimen (also Day21 represents accurately 4+14 days regimen).

**Figure 1. Timeline of the Study and related Measurements**

Two additional venous blood samples were taken 45 min after the non-acute samples at the Day0 and at the Day7 mornings. At the Day0, after the first blood sample there was on average 15 minute VO₂max test followed by the second venous blood sample exactly 30 min after finishing the VO2max test. At Day7, there was 45 min wait at rest and fasting before the second venous blood sample was drawn. In the morning at the Day7, the acute dose of DGA (3.33 mg/BW kg) or placebo mixed into water was taken immediately after the morning non-acute arterial blood sample. In addition, fingertip samples were taken at Day0 and Day7 with venous blood samples and an additional one immediately after the ending of VO₂max.

Selected doses, regimen times, and measurements in the present study were based on earlier in vitro and in vivo pilot pre-tests related to patenting [13] and regulatory acceptance processes of DGA.

Existence of the placebo group throughout the study was informed to the participants but in practice before the Day7 measurements the number of placebo-treated participants was zero. From the Day7 onwards, the number of participants in the placebo group was increased to 10, i.e. the placebo group was de facto introduced only for the 2-week follow up period.

**Test Substances and Doses, Preparation and Administration**

Administered D-glyceric acid calcium salt (DGAs) and placebo (E509 / calcium chloride) were mixed into 1.5 l bottles of water in beforehand. At Day0 and at Day7 (see Fig. 1), each person received calculated dose of DGA or placebo, mixed into water, to be drank in the morning and in the evening. In the placebo group there was equal molar amount of calcium with water. The purity of used DGAs batch was tested in Finnish Food Authority for residues, in VTT Technical Research Centre of Finland for DGA concentration, and Pharmatory Oy for enantiomers.

The effective dose of DGA was 2 x 3.33 mg/BW kg. In the 14-day follow up period the DGA regimen was reduced to half, i.e. 1.67 mg/BW kg twice a day (Fig. 1). Dose was reduced to half because we wanted to investigate dose dependence and sufficient dose for possible signaling effect of the DGA regimen.

**Blood Samples**

Blood samples were drawn from the antecubital vein from each participant always at the same time in the morning. The samples were immediately cooled and centrifuged in heparin plasma tubes and stored into 2 ml portions in -80 °C. Fingertip samples were collected into capillary tubes for analysing blood glucose (Fig. 4) and lactate during the same day in the Biosen C-line equipment (EKF Diagnostics). Venous samples were analysed in three different places: Nightingale Health Oy [14] (NMR metabolomics platform with
regulatory approval for diagnostics, Table 2: lactate, glycerol, and citrate; Table 3: glucose, TGs, total FAs, beta hydroxybutyrate, alanine, and GlycA; and in the VTT Technical Research Centre of Finland Gas Chromatography Mass Spectrometry (GC-MS [ 1, 2]) concentration measurements of DGA and lactate were conducted. When e.g. lactate was measured in several laboratories from the same samples, we could cross check the consistency of non-accredited GC-MS results obtained with different techniques.

**RNA sequencing**

Whole genome RNA sequencing from collected antecubital blood samples was conducted by the Institute for Molecular Medicine Technology Center’s Genomics Unit (Helsinki, Finland). Collection of non-acute whole blood samples into PAXgene Blood RNA Tubes and extraction of mRNA was conducted at the research site according to manufacturer’s instructions. Differential gene expression (DGE) analyses were conducted for nuclear and mitochondrial OXPHOS genes.

**Indirect VO₂max Test**

Submaximal and indirect VO₂max testing was selected due to unacceptable health risks related to maximal physical exertion in the 50-60-year old persons. This method also gives sufficient estimate of VO₂max for our purposes [ 15]. Starting workload was 50 W for females and 75 W for males. Workload was increased by 25 watts every 2 min and the pedal rate (PR) was kept fixed. Heart rate (HR) measurement was continuous. HR and the rate of perceived exertion (RPE) were recorded always when the workload was increased (scale from 6 – 20). Ending criteria: when she or he could not continue anymore and her or his HR reached close to estimated maximal value, or when PR declines below targeted fixed rate of 60-65 rpm and the participant was not able to reach the targeted PR anymore. All of the participants reached sufficient HR and RPE for the estimation of maximal oxygen uptake. The VO₂max estimation formula was based on maximal power produced in the end of the test and body mass [ 15].

**Data Ranking by the length of participants’ VO₂max test and aerobic capacity**

Only those who cycled at least 16 min were selected to the study that measured blood DGA content 30 min after the ending of the VO₂max test (Fig. 3A). The decision to choose longest cycled participants (4 males and 4 females) was done in advance and was based on assumed sufficient and homogenous workload. Blood DGA measurement was pretested with two homogenous (high aerobic capacity, 50-60 year old) male persons that cycled in minimum 16 min, i.e. similar workload (Supplement 1 a).

**Figure 2 A and B.** Cross plots of Day0 low-grade systemic inflammation (A) and TGs (B) and schematic division of the data into two halves by VO₂max (38 ml/kg/min) before any treatments of the present study.
On top of homogenous workload, ranking all the participants by aerobic capacity (AC) was essential and needed when studying possible therapeutic effects of the activation of EM. It is generally known that person’s AC and EM correlates strongly e.g. with subclinical inflammation and blood lipid profile (Fig. 2 A and B). When ranking the whole data individually by the Day0 VO$_2$max (Fig. 2 A and B), we were able to avoid arbitrary selection of persons into certain AC group. Nevertheless, based on general assessment independently of the present study, neutral clustering to subgroups based on participants’ AC before regimens in the study made scientifically sense. Based on [16] and obtained VO$_2$max results, we decided to classify “lower AC subgroup” and “higher AC subgroup” as the participants with Day0 VO$_2$max lower than or higher than 38 ml/kg/min, later the “LC subgroup” and “HC subgroup” respectively. No result was dependent on this arbitrary selection condition.

Our focus group was naturally the LC subgroup because it was generally known that the activation of the EM possessed greater positive potential in that group. Despite different health improvement potential between the LC and HC subgroups the signal for the EM activation was expected to be similar in both the HC and LC subgroups.

**Table 1. Characteristics of the Longest Cyclers, and the HC and LC subgroups at Day0**

| Subgroup | Longest Cyclers (N=8) | LC subgroup (N=18) | HC subgroup (N=9) |
|----------|-----------------------|--------------------|-------------------|
| Average VO$_2$max | 43.5 ml/kg/min | 31.3 ml/kg/min | 43.8 ml/kg/min |
| Average age | 54 years | 55 years | 56 years |
| Average BMI | 24.7 | 26.2 | 23.5 |
| Female / Male | 4 / 4 | 12 / 6 | 4 / 5 |

**Statistical Tests**

All blinded participants were in the same comparison group during the first week in order to maximize the number of observations for achieving statistically robust results. Presented statistical test results are always parametric and based on central limit theorem derived asymptotic normality. Additionally, all the tests are based on paired t-testing when possible. In the paired t-tests the same person’s measurement results are compared before and after the treatment, i.e. we used each participant as her own control. All presented paired t-tests were predetermined or are directly derived from predetermined comparisons. P-value lower or equal to 0.05 in a one-sided t-test was considered statistically significant and p-value lower or equal to 0.01 as statistically very significant.

In the LC versus HC subgroup comparisons in 4-days, and also in comparisons with the DGA and placebo groups at Day21, we were forced to use less efficient unpaired t-testing. Nevertheless, we have used intraindividual changes to calculate group average changes for the test variable. This increases the statistical power of the test. For the avoidance of doubt, all statistically significant Day21 parametric tests (DGA compared to placebo) were verified with non-parametric Mann – Whitney- Wilcoxon U -test. This was done because the number of observations in the placebo group was only 10. Non-parametric tests gave same statistical relevance levels as parametric tests.

Squared linear correlation (R$^2$) has been calculated in Figs. 3, A and B, 5 B, 7 A and B. R$^2$ can be interpreted as the ratio of “explained” variation to the total variation of the explanatory variable, e.g. persons’ GlycA explained by VO$_2$max in Fig. 2 A. Correlations do not show causalities but in certain situations main direction of causality can be deducted from earlier findings and reasoning, e.g. R$^2$ = 0.425 in Fig. 2 A indicates that VO$_2$max “explains” 42.5% of the total variation in GlycA at Day0. This is a very strong and generally known relation. Significance of R$^2$ can be statistically tested by F-test with 1 and 23 degrees of freedom, ANOVA for regression [17].
Results

Endogenous increase in plasma DGA after strenuous exercise

The endogenous DGA concentration in plasma increased statistically significantly 30 min after the end of VO2max test in the pre-selected homogenous subgroup (Fig. 3 A), more information on the subgroup selection for this test can be found in Materials and methods.

**Figure 3 A and B.** Endogenous plasma DGA before and 30 min after the end of VO2max test at fast (A). Endogenous plasma DGA after fasting for 45 min (B). In both conditions, the time between measurements is roughly 45 min.

To control that the increase in fasting DGA does not arise due to some circadian or other unrelated effect, we measured fasting DGA also without VO2max at Day7. In Fig. 3 B we can see that prolonged fasting does not pose any significant circadian effect on plasma DGA (3.96 vs. 3.91 umol/l after 45 min in placebo). There does not seem to be any circadian effect in endogenous DGA thus we can conclude that strenuous exercise, like VO2max cycling with sufficient workload, seemed to increase the plasma DGA concentration.

Glucose supply from the liver after VO2max and after exogenous DGA resemble each other, response of the DGA deviates from the placebo

On top of measuring aerobic condition of the participants at Day0, the VO2max test was conducted to compare acute responses of a bout of strenuous exercises to the response of an acute dose of DGA at Day7. In both observation points at Day0 and Day7 the time between non-acute and acute measurement was approximately 45 min (Fig. 1).

**Figure 4 A and B.** Plasma fasting glucose before and after VO2max at Day0 and 45 min rest at Day7 (A), and 45 min millimolar changes in plasma glucose at Days 0 and 7 (B).
Figure 4 A presents plasma glucose levels from non-acute (Pre) to acute (45 min after) measurement on Day0 and on Day7. The generally recognized, but limitedly studied, phenomenon that plasma glucose level increases during and after VO\(_2\text{max}\) (solid line) can be clearly seen (p=0.028). Further, it can be seen that 45 min prolonged “Fasting + rest” resulted in moderately declined blood glucose level (dotted line). Figure 4 B shows that in a one-sided pairwise t-test the response to acute DGA dose could not be statistically differentiated from the response to VO\(_2\text{max}\) in the same persons (P=0.16) and simultaneously the placebo group differs from both DGA and VO\(_2\text{max}\).

**4-day DGA regimen possessed indirect indications of exercise effect**

At Day0, i.e. before any DGA regimen, plasma TGs and subclinical inflammation (GlycA) were lower in the HC subgroup compared to the LC subgroup (Figs. 2 A and B). These differences were statistically significant (Table 3). Furthermore, downward sloping trend in GlycA and TGs with increasing AC in Figs. 2 A and B are generally known phenomena. The 4-day DGA regimen improved GlycA statistically very significant in the LC subgroup (Table 3). Also, elevated plasma TGs in the LC subgroup were reduced strongly and rapidly towards recommended 1 mM concentration under the 4-day DGA regimen (Table 3).

To avoid possible biases due to artificial classifications, we also cross plotted the %-changes of GlycA and TGs under the 4-day DGA regimen and Day0 VO\(_2\text{max}\) result individually (Figs. 5 A and B). We furthermore tested statistical significance of VO\(_2\text{max}\) (AC) as the explanatory variable for possible trends in the GlycA and TGs changes under the 4-day DGA regimen. In both cases increasing AC could explain rising trend statistically very significantly measured by \(R^2\) (Figs. 5 A and B).

**Figure 5 A and B.** The relationship between 4-day % -changes of GlycA (A) and TGs (B) and VO\(_2\text{max}\) under the DGA regimen.

![Graph A](image1)

![Graph B](image2)

**Energy substrates for aerobic glycolysis, gluconeogenesis, and triglyceride synthesis**

Plasma concentrations of lactate, citrate, and glycerol all decreased statistically very significantly during the 4-day DGA regimen (Table 2). In Supplement 2, the 4-day changes and starting concentrations of lactate, citrate, and glycerol are presented and ranked individually for each participant by VO\(_2\text{max}\). For lactate also by Day0 starting concentration (Supplement 2, Fig. E).

**Table 2.** Average Day0, Day7 and Day21 concentrations of plasma metabolites related to mitochondrial energy production, gluco-/glyceroneogenesis, and FA synthesis (non-acute, last dose previous night)
How were other energy metabolic substrates affected in non-acute resting and fasting conditions?

Glucose and total FAs form clear majority of energy metabolic substrates in blood circulation. Total FAs include also fats packed as TGs. During the 4-day DGA regimen plasma glucose, TGs, and alanine were on average reduced by 3-5%, and plasma total FAs excluding TGs and beta-hydroxybutyrate declined by 1-2% (Table 3). Notably, in plasma TGs there existed exceedingly clear difference on the starting concentrations between the LC and HC subgroups (Table 3) leading to totally different responses under the 4-day DGA regimen.

Table 3. Additional energy metabolic substrates and related plasma metrics (non-acute, last dose previous night)

| Metabolite / Biomarker | Day0/ 0-control (N=25) | Day7 / 4 Day Treatment (all DGA, N=25) | Day21 Group average, mM |
|------------------------|-------------------------|----------------------------------------|-------------------------|
|                        | Average, mM (std.)      | Average (mM) after 4-day DGA regimen (std.) | P-value in paired t-test vs. 0-control | % dose DGA (N=16) | Placebo (N=9) |
| Lactate, mM            | 1.05 (0.46)             | 0.84 (0.21)                             | 0.001                   | 0.84 *)          | 0.89 |
| Citrate, mM            | 0.131 (0.021)           | 0.122 (0.019)                           | 0.005                   | 0.132 #)         | 0.121 |
| Glycerol, mM           | 0.074 (0.028)           | 0.059 (0.019)                           | 0.008                   | 0.069 #)         | 0.065 |

Bolded numbers in the Tables 2 and 3 indicate statistical significance. Precise p-values are presented only for Day7 changes from Day0. For Day21 changes from Day0 following marks are used to indicate significance: statistically very significant = ***, and statistically significant = *) in 1-sided paired t-test. #) indicates statistically significant increase (retrieval) from Day7 concentration. ###) indicates statistically significant difference in the change from Day0 to Day21, DGA compared to placebo. Finally, ####) or #####) indicate statistically significant or very significant difference in the change from Day0 to Day21 when the LC subgroup was compared to the HC subgroup.

Day21 responses in Tables 2 and 3 are analysed in the chapter “Day21 metabolic considerations” of the Discussion section.

OxPhos mRNA expressions were modulated strongly in 4-days

Next, we measured global mRNA expressions from collected white blood cells at Day0, Day7 and Day21, all non-acute samples. Detailed results are presented in Supplement 3. Strong modulation of certain RNA expressions showed that the 4-day DGA regimen possessed an impact on aerobic OxPhos genes in the short term, and that mitochondrially coded genes were all upregulated at Day21 in the DGA regimen (Supplement 3). Simultaneously received important information was that the DGA regimen directly or indirectly possessed an energy metabolic impact also on the cells of the immune system. Further analyses on the modulation of the cells forming integral part of the immune system were beyond this article.
Discussion

Exogenous DGA regimen seemed to activate cellular energy production from all sources (glucose/lactate/pyruvate, FAs, amino acids, and ketone bodies). From in vivo plasma samples we can reliably and exactly measure only the net effects for metabolites at the time of the measurement. Nevertheless, in our meticulously planned test setup with VO$_2$max test, we were able to gain deeper understanding of the study group and compare the effects of the DGA regimen from multiple angels. Remarkably, the DGA regimen seemed to induce similar effects than strenuous bout of exercises without any noticeable increase in physical activity in all measurement points, 45 min, 4-days and even in 14-day follow-up DGA regimen. We could even conclude that some of the effects were clearly dependent on participants’ AC.

45 min acute test

Plasma glucose formed almost an ideal test marker to compare acute responses when fasting was prolonged by 45 min from the non-acute measurement. Our test setup made it possible to compare the DGA and placebo groups against each other, and importantly to pairwise test the glucose changes in the same persons at Day0 (VO$_2$max and fasting) compared to Day7 (fasting only). In the DGA group plasma glucose declined less than in the placebo (Fig. 4 B). In this test setting, the acute DGA dose seemed to initiate blood glucose maintenance that could not be differentiated from a bout of strenuous exercise (p=0.16), and at the same time the placebo group deviated statistically very significantly from exactly the same exercise effect (p=0.002). Furthermore, the acute response in the DGA group deviated significantly from the placebo group (p=0.03).

The liver starts supplying glucose to the blood stream immediately after initiating exercises [12, 18]. Instant activator of the liver is unknown, but initially it seems to be independent of (autonomic) innervation [12]. Tight hormonal regulation of glucose homeostasis follows very rapidly thereafter. Secured supply of glucose is vital e.g. for the functioning the brains. Glycogenolysis is the first source of hepatic glucose followed by gluconeogenesis, which requires a lot of energy that is primarily supplied by hepatic FA oxidation. Also muscular TGs are used at an increasing rate during physical exercises [19] and their use also facilitates glucose maintenance in plasma. Parallel process of restoring muscular energy stores after strenuous exercises, e.g. escaping a lion, was even lifesaving in ancient times supporting evolutionary fortification of rapid energy storage replenishing.

The DGA administration seemed to create an effect that was sufficiently strong to cause the liver to start providing glucose at an accelerated rate compared to the placebo. By flux calculations we excluded the possibility that extra glucose could have been metabolized from administered exogenous DGA. The DGA dose was more than 10-times too low for that. In Supplement 1 b, we have also excluded the possibility that random selection of the placebo group could have caused the differences observed in the Fig. 4 B. There still existed an unlikely explanation for the relative increase in glucose compared to the placebo. It was that the peripheral tissues somehow did not use glucose at the same rate under the DGA regimen compared to the placebo. In that case there did not have to be any relative increase in hepatic supply of glucose to obtain observed elevated plasma level. However, strong decline in lactate after the 4-day DGA regimen (Table 2) and also the relative decline in lactate compared to placebo in this acute test, +0.12 mM DGA and +0.28 mM placebo 45 min after the acute dose, pointed to the direction that aerobic glycolysis was activated, not deactivated, under the DGA regimen. Furthermore, also the accelerated ethanol oxidation [8] strongly supports the activation of hepatic glucose supply to peripheral tissues after acute DGA dose 1 hour earlier.
All in all, we can conclude that random group differences or inhibition of glycolysis did not cause the fact that acute DGA dose induced effects on blood glucose that differed from the placebo but could not be distinguished from a bout of exercise like the placebo clearly could.

**Activation of EM in 4-days**

During the first 4-days the study participants received altogether 8 doses of DGA. In stable conditions, the only sufficiently covering explanation for the remarkable changes in lactate, citrate, and glycerol (Table 2) was that the cellular and tissue metabolism of these substrates had increased. This increase in metabolism caused a decline in their intracellular concentrations, which further caused observed influx of these substrates from plasma to balance plasma membrane concentration gradients. Lactate, citrate, and glycerol are all directly or indirectly and primarily used in cellular aerobic EM. Thus, it seemed that the DGA regimen remarkably activated aerobic EM at the whole body level in already 4 days. Parallel information on the 4-day changes in plasma glycerol (-21%, p=0.008) and citrate (-7%, p=0.005) clearly suggest that on top of activated EM also increased syntheses of TGs and FAs seemed to materialize during the 4-day DGA regimen compared to Day0, [20, 21, 22, 23, 24, 25]. Furthermore, also the 4-day improvements in blood TGs and GlycA point to repeated activation of overall EM.

Blood TGs in circulation are not regulated at a certain tight concentration range like glucose (Table 3). At Day0, there was statistically very significant difference in plasma TGs (p=0.007) between the LC subgroup (1.21 mM) and the HC subgroup (0.73 mM). At Day0, also average GlycA differed statistically significantly (p=0.014) between the HC and LC subgroups (Table 3). Low intra-individual variability and its predictive ability in healthy individuals [18, 26] were the reasons why GlycA (Fig. 2 A) was chosen as the marker for low-grade inflammation [27]. Furthermore, subclinical inflammation is on average somewhat elevated already in 50-60-year old apparently healthy persons [28].

Initiating physical “exercise regimen” improves AC, lipid profile and subclinical inflammation markers. This is especially evident in persons who do not conduct high levels of exercises like in the LC subgroup. The 4-day DGA regimen improved GlycA statistically very significantly in the LC subgroup (Table 3). Also, elevated plasma TGs in the LC subgroup were reduced strongly and rapidly towards recommended 1 mM concentration under the 4-day DGA regimen (Table 3). These 4-day improvements in the LC subgroup were very favorable and at the same time they moved average TGs and GlycA towards levels that indirectly indicate (Figs. 2 A and B) an improvement in the average AC, although any actual improvement in AC probably did not happen at least in any meaningful amount.

Nevertheless, we can conclude that under the 4-day DGA regimen overall EM was cumulatively strongly activated, like seemed to be the case also in 45 min one dose experiment.

**EM homeostasis**

When EM is activated like with physical exercises, the liver starts immediately providing energy substrates to peripheral tissues like muscles (Fig. 4 A) [30]. Fast activation of whole body EM by the DGA administration was initially chosen as the main objective of our studies. This was because, when implicit assumption of predominantly hepatic metabolism of DGA was abandoned, that explanation was clearly the most likely cause of observed, very surprising 25% enhancement of alcohol oxidation in rat livers in [8]. Glucose and other energy substrate supply to peripheral tissues consumes a lot of energy. According to novel indirect flux calculation, the DGA administration 1 hour before ethanol in that study unintentionally created energy deprivation in the livers that further made enhanced ethanol oxidation possible. The abnormally high ethanol oxidation rate produced high energy NADH molecules at faster than normal pace because it facilitated fast recovery of energy homeostasis to the livers.
Real consumption of energy substrates at rest cannot increase very substantially and energy is not produced in excess of demand. Nevertheless, a surprisingly strong effect seemed to materialize towards EM and in related energy metabolic substrates (Table 2). Also the 4-day changes in main energy substrates (Table 3) were in line and supported the hypotheses of activated energy consumption due to the DGA regimen. Likely explanation was that the DGA-signal activated on top of overall EM also tissue energy stores replenishing, like happens during and after physical activity. On top of glucose for glycogen stores, also the transportation and storage of TGs were likely activated. Gluconeogenesis, FA synthesis, and other anabolic reactions consume a lot of energy. Additionally, also intracellular quality control of protein folding and unfolding may consume available extra energy [29].

Aerobic glycolysis and FA oxidation typically compete with each other because the end-product for both in mitochondrial matrix is the same (acetyl-Coa). It seemed that the DGA-signal at least temporarily overwhelmed this “competition” at whole body level. The explanation may be that depending on the tissue type the DGA-signal favors preferred way of producing energy fast in each tissue type.

**Day21 metabolic considerations**

At Day21, lactate remained 19 % lower than at Day0 in the DGA group and the change was statistically significant, whereas in the placebo group lactate returns closer to the initial value and the difference was not any more statistically significant (Table 2). Interestingly, plasma glycerol increased statistically significantly from Day7 onwards to Day21 in the DGA group (Table 2). Observed rise in glycerol in the DGA group was 19% compared to the respective change of 6.5% in the placebo group. In line with plasma glycerol, also plasma citrate concentration at Day21 was returned back to the Day0 level in the DGA treatment group but not in the placebo group (Table 2). Citrate is produced in the TCA in mitochondrial matrix, thus more rapid return may indicate a difference in the activity of mitochondrial EM between the DGA and placebo groups.

At Day21, plasma glucose was slightly higher in the DGA group compared to the placebo. The difference was small but nevertheless it was in line with the observation that DGA regimen promotes glucose homeostasis. Additionally, improved blood lipid profile was observed in the DGA group but not in the placebo at Day21. Plasma alanine continued also to be down at Day21 in the DGA group. Reduction in alanine was likely due to its use as an energy metabolic substrate via pyruvate in the liver.

The difference at Day21 in beta-hydroxybutyrate (BHB) compared to the placebo indicated that the DGA administration enhanced liver function and/or increased mitochondrial metabolism (Table 3). **BHB is a ketone body that is synthesized in the liver from FAs and it represents an essential carrier of energy from the liver to peripheral tissues when the supply of glucose is too low for the body’s energetic needs, such as during periods of prolonged exercise [31]. BHB is the most abundant ketone body in mammals and functions also as an important signaling molecule that e.g. suppresses excessive ROS [32, 33]. Consumption of succinyl-CoA during BHB utilization as a mitochondrial energy substrate and consequent reduction in mitochondrial protein succinylation may regulate many of the crucial mitochondrial pathways in peripheral tissues, perhaps favoring a shift to more lipid-dependent energy usage [31]. The statistically significant increase and deviation in BHB from the placebo was in line with the hypothesis that the DGA regimen induced some kind of “exercise signal effect” and that this it continued in the 14-day follow up period with the halved DGA dose.**

**Health implications**

It was very reasonable that TGs and GlycA seemed to improve only in the LC subgroup, who possessed inferior initial levels. Participants in the HC subgroup possessed exceptionally high AC compared to international rankings [16] of the same age group and thus there was no reason to expect that their AC
related health parameters would improve further either by adding more exercises or due to the DGA regimen. Improved lipid profile and activation of hepatic function are important. Furthermore, the combination of enhanced aerobic ATP production and simultaneous reduction of subclinical inflammation may cause significant pleiotropic therapeutic effects at the whole body level for those in need. This kind of non-stressful enhancement of aerobic energy production may resolve energy metabolic challenges in active tissues and main organs.

**Putative explanation for the endogenous increases in plasma and intracellular DGA concentration**

In high energy demand situation glycolysis is activated to a degree where mitochondrial capacity to buffer proton (H+) release from glycolysis and ATP hydrolysis, and simultaneously to provide sufficient NAD+ for maintenance of high glycolytic ATP production is temporarily exceeded [35]. In this kind of a situation the flow of pyruvate into mitochondrial TCA cycle from glycolysis is redirected towards lactate (black bar in Fig. 6). Lactate dehydrogenase reaction consumes cytosolic H+ and provides NAD+ thus it assists in maintaining rapid glycolytic ATP production and even the use of ATP [35]. Nevertheless, lactate accumulation is not sustainable, and the situation leads to slow down in glycolysis and to buildup of glycolytic intermediates like 2-phosphoglycerate. DGA and D-GALD flows towards glycolysis are exceedingly slowed down (grey bars in Fig. 6). Intracellular DGA starts to accumulate fast.

**Figure 6.** Schematic illustration of the cellular metabolism of DGA in high energy demand situation.

Abbreviations: MCT (monocarboxylate transporter), IMS (intermembrane space), GLUT, GLUT5 and GLUT11 (glucose and/or fructose transporters), GLYCTKm / GLYCTKc (mitochondrially or cytosolically located glycerate kinase splice variants), ALDHm / ALDHc (mitochondrially or cytosolically located glyceraldehyde dehydrogenase, in muscles e.g. ALDH1B1 [5]), D-GALD (D-glyceraldehyde), DGA-SIGNAL (“activate aerobic mitochondrial ATP production and related antioxidant pathways”), G3Psh (glycerol phosphate shuttle [36]), GRHPR (glyoxylate reductase hydroxybutyrate reductase), DGA-HPA -enzyme loop (enhanced maintenance of cytosolic redox ratios), HPA (hydroxybutyrate), TCA (tricarboxylic acid cycle), and FA oxid. (fatty acid oxidation). Human enzymes are mostly from KEGG database (www.genome.jp/kegg/).

Skeletal muscles and many other organs possess GLUT transporters that are specified into fructose intake from plasma (Fig. 6). Millimolar (0-1.0 mM) plasma concentration of fructose, that are roughly 100 times the average plasma DGA concentration, suggests that ALDH derived production of DGA (Fig. 6) is the dominant source of intracellular DGA in tissues [6, 7]. It is likely, that in high energy demand situations
intracellular concentrations of DGA rise temporarily to much higher levels than in plasma in any physiological condition. Fructose intake by muscle cells and its partial conversion into D-GALD, makes skeletal muscles the most likely main source of increased plasma DGA concentration (Fig. 3 A) after exhaustive exercise. DGA concentration increases also in other tissues and organs that face high energy need during intense aerobic exercises like cardiovascular, lungs, gluconeogenic organs, and at least partially also the nervous and immune systems. Simultaneous intracellular DGA upregulation in all these organs and tissues enables whole body coordination, even without paracrine or endocrine regulation. Intracellular DGA-signal may be somehow mediated via certain enzyme splice variant but probably the full signaling cascade consists of multiple locations, enzymes, receptors etc. There may be also endocrine DGA-signaling via plasma but modest only some 11% increase in micromolar plasma concentration makes it unlikely or in any case secondary.

Endogenous DGA-signaling

An increase in the endogenous plasma DGA concentration was observed after the strenuous bout of exercise (Fig. 3 A). In our study setup, this can only be due to the increase in intracellular DGA synthesis (more information on the study setup in Supplement 1 a). Endogenous intracellular DGA increase possibly gives cells a warning, the DGA-signal, that cytosolic energy production (glycolysis) is approaching full capacity.

Based on received results from present study endogenous DGA-signaling does not seem to be considerably robust but exogenous DGA regimen provides surprisingly strong results. This raises questions on the origin of the DGA-signaling in the evolution of cellular EM. Has it been somehow active already before the times of acquiring mitochondria and the dominance of present type of glycolysis ending to pyruvate? Without mitochondria an ancient, possibly prokaryote [37], cell could produce ATP faster when glucose catabolism ended to DGA via GLYCTK compared to pyruvate. We do not know but based on current results the DGA-signaling has “remained” and/or evolutionarily developed to be part of the overall multifaceted physical exercise signal transduction cascade at whole body level. Current advantage relies on additional signaling possibilities that facilitate faster adaptation to higher exercise intensity. Further research on the DGA-signaling cascade is needed.

Conclusions

Based on the results, we hypothesize that exogenous DGA creates an intracellular signal or a cascade of signals “more ATP is needed immediately”, similar to when conducting strenuous exercises. Both tested exogenous DGA doses were exceedingly small compared to caused metabolic effects. Additionally, the reduction of the DGA dose to half did not seem to lessen the efficacy. Thus, we may conclude that the effects must be based on some indirect impacts, like signaling.

The effects of exogenous DGA seemed to last throughout the 14-day follow up period with halved dose. It seems possible that the DGA regimen may alleviate many chronic disorders that are caused by elevated systemic inflammation and/or lipid profile. Enhanced whole body aerobic EM and reduced systemic inflammation are important inter alia in the liver, kidney, muscular and cardiac diseases, and metabolic disorders related to obesity. Whole body mechanism of action points especially to the alleviation of fatty liver disease but all malfunctions and illnesses that can be ameliorated or postponed by increased physical exercises may be possible targets of the DGA therapy.

Declarations
Ethics approval and consent to participate

All the participants were informed of the experimental design, and the benefits and possible risks that could be associated with the study prior to signing an informed consent to voluntary participate in the study. The studies were conducted in line with the statement of the Ethical committee of the Central Finland Health Care District (KSSHP).

Consent for publication (not applicable)

Availability of data and materials

See IPD sharing statement in ClinicalTrials.com (NCT04713319). Characteristics of the main data on which the conclusion of this paper rely are also presented in the Supplement 2. Main data used and/or analysed during the current study are available from the corresponding author on reasonable request.

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Author Information and Contributions

Prof. Heikki Kyröläinen was the study director and specialists in exercise related human physiology. Prof. Heikki Kainulainen was the expert in the physiology of energy metabolism at cellular and enzyme level. Mr. Kainulainen was also the expert in FA/TG synthesis and oxidation in muscles and at whole body level. Corresponding author, O. Petteri Hirvonen, did all the statistical analyses and planned the study setup from that point of view. Mr. Hirvonen has also been involved in extensive pilot studies that established predetermined guidelines for the timelines and the doses for the present study.

Conflict of Interest

Scientifically there are no conflicts of interests. For 8 years, the DGA molecule has been successfully tested by independent methods and often outsourced organizations as a potential therapeutic agent by Replicon Health Oy (RH). Petteri Hirvonen is the research director and a major owner of RH. RH owns several patents related to the use of DGA. Heikki Kainulainen also owns very minor number of shares in RH.

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Supplement 1 a and b

Supplement 1 a. Pilot VO₂max Study

VO₂max test setting was independently pre-tested with two 50-60-year-old males who could cycle at least 16 min with increasing load towards exhaustion. The idea in the pilot was that timewise sufficient workload in a strenuous bout of exercise could significantly facilitate possible increase in endogenous DGA in plasma. Pre-test gave results (Graph 2 B) and that was why the homogenous “longest cyclers” subgroup (N=8) was chosen to the present study. (Longer term exercise may work better in revealing endogenous DGA increase but that would be clearly more challenging to arrange in strictly controlled circumstances.)

Graph 2 A and B. Comparison of indexed plasma DGA concentrations, A = present study, and B = pilot study

In Graph 2B, we have named the pre-tested persons as DGA1 and DGA2. The results on the plasma DGA were remarkably similar than in the present experiment. Nevertheless, the increase in the plasma DGA 30 min after the end of VO₂max was slightly higher in the pilot study. This was probably caused by the low number of participants in the pilot. (Graph 2 A is the same as Fig. 3 A in the article except that the values are indexed so that “Pre” results are 100. Indexing helps the comparison to Graph 2B.) In the pilot, also an immediate sample from blood was taken immediately after the end of VO₂max test (see “Post” in Graph 2B). It showed modest only 5-10% increase in plasma DGA, and some ten-fold increase in lactate in both tested (not shown). The lactate increase was similar as in present study (not shown).

Supplement 1 b. Day0 glucose concentrations and the 4-day changes in the placebo and DGA subgroups

Paired test setting in the DGA and placebo groups compared to VO₂max made these acute test result reliable but there was a possibility that some unlikely random variation may have caused differences to the initial DGA and placebo group selection that further non-intentionally caused the differences observed in the Fig. 4 B. In the following we analyze and aim at ruling out this possibility. Starting Day0 glucose values in the DGA and placebo groups differed somewhat (4.2 mM and 4.0 mM) but the difference was not even close of being statistically significant. Due to somewhat different starting values the group responses 30 min after VO₂max differed marginally (4.4 mM and 4.7 mM) but also that difference in glucose was not significant. Furthermore, the 4-day changes under the DGA regimen in each subgroup did not differ statistically significantly (-4.6% and +2.6%) but interestingly and in line with glucose homeostatic requirement the levels in glucose after the 4-day DGA regimen moved marginally closer each other 4.05 mM (the DGA group) and 4.10 mM (the placebo group). Additionally, neither of the pair wised tested subgroups differed during the 4-day DGA regimen from starting values (p-value in 1-sided t-tests were 0.144 and 0.177).
**Supplement 2.** Figures A, B, C, and D. The Day0 starting concentrations (rh scale) and the 4 day %-changes (lh scale) of lactate (A), glucose (B), citrate (C) and glycerol (D) under the DGA regimen. Data is ranked from the highest to the lowest VO\(_2\)max. No dependence at Day0 or in 4-day changes with VO\(_2\)max existed, i.e. no difference between the HC (1-9) and LC (10-27) subgroups existed.

**Figures E and F.** Changes in plasma lactate (E) and triglycerides (F) during the 4-day DGA regimen ranked by starting Day0 concentration. Linear dependence (dotted lines) showed that the higher participant’s Day 1 level was, the bigger the decline during the 4-day DGA regimen, and vice versa. Remarkably strong homeostatic effect on especially lactate can be seen also in the standard deviations in Table 2.
Supplement 3.

**OXPHOS mRNA expressions were modulated strongly in 4-Days and upregulated in 21-days**

mRNA expressions were measured from collected white blood cells at Day0, Day7 and Day21, all non-acute samples. Due to multiple reasons the mRNA expressions do not talk much about changes in real enzyme activities even for the specific gene in question. Nevertheless, possible short modulations and long-term trends in basal mRNA activity, as well as possible HC and LC subgroup differences may possess valuable information like in the case of ATPIF1. ATPIF1 gene RNA expression was modestly, only 3%, but statistically extremely significantly (p=0.0004) reduced in the LC subgroup (Table 4), whereas in the HC subgroup there was a small but statistically not significant +1% upregulation on average.

**Table 4.** Strong modulation in OXPHOS genes at Day7 and paired change from Day0 to Day21 in the DGA and placebo groups. **Figure G.** 3-week changes of mitochondrially coded OXPHOS genes.

| OXPHOS genes | Day0 | Day7 | Day21-Day0 |
|--------------|------|------|------------|
| ATPsynthase (Inner membrane gradient consuming or regulating) |
| F$_0$F$_6$ subunit | 4.36 | 4.13 | 0.007 | -0.02/+0.21 |
| F$_1$alpha subunit | 1.99 | 2.26 | 0.045 | -0.04/+0.08 |
| ATPIF1 LC subgroup | 5.43 | 5.27 | 0.0004 | N/A |
| MT-ATP8 ATP synth. | 4.34 | 4.11 | 0.037 | +0.17/+0.02 |
| Inner Membrane Gradient Activators (IMGAs) |
| MT-NDs (C I) | 11.59 | 11.49 | 0.16 | +0.12/-0.02 |
| MT-CYB (CII) | 10.30 | 10.22 | 0.26 | +0.20/+0.05 |
| MT-CO1 (C IV) | 8.88 | 8.54 | 0.19 | +0.17/-0.06 |
| Combined Mitochondrially coded MT-enzymes (MT-ATP6 also included) |
| MTs combined | 8.46 | 8.32 | 0.08 | +0.16/+0.017 |

Bolded numbers in the Table 4 indicate approximate statistical significance. Error bars in Fig. G are calculated from 3-week changes in the DGA group (standard error of the mean).

Robust RNA modulations after the 4-day DGA regimen materialized in certain F$_1$F$_0$-ATP synthase complex (ATPsynthase) enzymes. E.g. gene F$_6$ in the “F$_0$ subunit” was clearly downregulated but the “F$_1$ subunit” gene alpha was upregulated after the 4-day DGA regimen (Table 4). Differing but statistically significant responses in F$_0$F$_6$ and F$_1$alpha enzymes were likely due to stability requirement in ATP production by the ATPsynthase at rest and fasting. Statistically extremely significant downward modulation of ATPIF1 gene in LC subgroup supports homeostatic modulation (Table 4) but more research is needed. ATPIF1 is believed to prevent ATPsynthase from switching to ATP hydrolysis during collapse of the electrochemical gradient [34]. F$_0$F$_6$ (and F$_0$B) possessed the highest nuclear RNA signal among F$_0$ subunit genes by a wide margin and similarly F$_1$alpha and ATPIF1 were the highest among F$_1$ subunit nuclear genes (altogether some 20 genes).

In Table 4, mitochondrially coded genes begin with letters “MT”. MT-NDs represent Complex I, and MT-CYB Complex III, and MT-CO1 Complex IV. Additionally, MT-ATP8 represent two mitochondrially encoded genes (ATP8 and ATP6) that relate to ATPsynthase. OXPHOS complexes pump protons to the intermembrane space (IMS) and activate inner membrane gradient and thus are shortened as IMGAs. All MT genes were modulated down at Day7 but typically this modulation was not statistically significant at individual gene/enzyme level. Interestingly all MT genes were upregulated compared to the placebo at Day21 (Table 4). When combined it was possible to form a statistical test variable that shows clear upregulation in combined MT genes at Day21 in the DGA group but not in the placebo group (Fig. G).