In this issue: Improving the preanalytical phase in laboratory medicine

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Can chewing gum be another source of preanalytical variability in fasting outpatients?

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ABSTRACT

Introduction

In the daily laboratory practice, there are patients coming to blood collection sites chewing sugar-free gum, considering it irrelevant to laboratory tests. The aim of this study was to evaluate whether a sugar-free chewing gum can interfere with laboratory tests.

Methods

We studied 22 healthy volunteers. After a 12-hour overnight fasting, the first blood sample was collected between 8:00 and 8:30 a.m. Then, immediately after the first venous blood collection, the subjects started chewing the gum (declared sugar-free) for 20 min. Subsequent venous blood samples were collected at 1, 2, and 4 hours after chewing the gum. Significant differences between samples were assessed by the Wilcoxon ranked-pairs test.

Results

Among all the results, statistically significant differences ($p < 0.05$) between basal and x hours after chewing

sugar-free gum were observed for the following parameters: cortisol, insulin, C-peptide, triglycerides, uric acid, urea, amylase, alanine aminotransferase, lipase, creatine kinase, total bilirubin, direct bilirubin, phosphate, iron, potassium, thyroid stimulating hormone, red blood cell count, hematocrit, hemoglobin, mean cell volume, red cell distribution width, white blood cell count, lymphocytes, neutrophils, and eosinophils; whereas, coagulation tests were not impacted by chewing sugar-free gum.

Conclusions

We recommend instructing the patients to avoid the use of chewing gum before blood collection for laboratory tests.

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INTRODUCTION

Chewing is essential during food consumption since it helps swallowing and food digestion. Moreover, Hirano et al. have shown that chewing is linked with cognitive functions (i.e., learning, memory and attention) [1]. Regarding the prolonged chewing consequent to chewing gum habit, it has some favorable outcomes such as: i) removal of food debris and plaque from teeth; ii) stimulation of saliva flow; and iii) reduction of gingivitis [2]. Moreover, chewing gum has been proven to stimulate the cephalic phase of gastric secretion on par with food chewing. The amount of acid output stimulated after 15 min of chewing the gum was very similar to the acid output after a cheeseburger meal [3]. It is well known that the hydrochloric acid secretion into the stomach is linked to:

1) extraction from blood plasma of Cl^-, Na^+, CO_2, and H_2O;

2) maintenance of blood electrolyte equilibrium through the release of HCO_3^- into the blood leaving the stomach (alkaline tide) [4].

Therefore, the fake feeding-induced gastric secretion due to chewing gum should be considered carefully when laboratory tests needing patient fasting are to be performed.

Currently, the management of patient preparation before laboratory testing requires attention since the evidence has shown that fasting time is required before blood sampling [5-7], except for lipid profile assessment [8, 9]. However, the GP 41, a standard for global application developed through the Clinical and Laboratory Standards Institute (CLSI) consensus process, recommends *"no chewing gum, or other objects (eg. thermometer) should be in the patient's mouth at the time the specimen is collected"* although no information is provided about allowance or denial regarding chewing gum habit before blood specimen collection [10]; whereas, a joint document from the European Federation of Clinical Chemistry and Laboratory Medicine (EFLM) and Latin America Confederation of Clinical Biochemistry (COLABIOCLI) states that "chewing gum should not be used" without any scientific evidence that it could be a source of laboratory variability [11].

In the daily laboratory practice, there are patients coming to blood collection sites chewing sugar-free gum, considering it irrelevant with respect to fasting status. Moreover, laboratory professionals lack evidence to define patient preparation regarding this issue. The aim of this study was to evaluate whether chewing sugar-free gum can interfere with laboratory tests.

MATERIALS AND METHODS

A total of 22 healthy volunteers (13 women and 9 men; average age was 31 (22-52) years) were selected from the personnel of the Center of Laboratory Medicine of the Vilnius University Hospital Santaros Klinikos and included in the study. Informed consent was obtained from all study subjects according to the 2013 Declaration

of Helsinki and the protocol was approved by the Ethic Committee.

After a 12-hour overnight fast, the first blood sample was collected between 8:00 and 8:30 a.m. Then, immediately after the first venous blood collection, the subjects started chewing 2.8 g of sugar-free chewing gum (Orbit Spearmint, Wrigley company, Plymouth, USA) for 20 minutes. The composition of the chewing gum is declared in Table 1. Subsequent venous blood samples were collected at 1, 2, and 4 hours after chewing sugar-free gum.

Table 1 Nutritional composition of sugar-free chewing gum

Nutritional composition	
overall weight (g)	2.8
KJ	17.8
Kcal	4.3
total carbohydrates (g)	1.8
proteins (g)	0
total lipids (g)	0

According to the CLSI GP 41 and the EFLM-COLABIOCLI recommendations [10, 11], all venous blood sampling procedures were carried out by a single phlebotomist. In order to eliminate possible blood distribution interferences, all volunteers were kept in an upright sitting position for 15 min [12-14]. Then, a vein was located on the forearm using a subcutaneous tissue transilluminator device (Venoscópio IV plus; Duan do Brasil, Brazil), in order to prevent venous stasis interference through tourniquet [15-18] and to avoid clenching [19].

All blood samples were collected directly into one 1.8 mL evacuated tube containing 3.2% sodium citrate 9N, one 3.5 mL evacuated tube containing gel separator and clot activator for serum samples, and one 4.0 mL evacuated tube containing K_2EDTA (Vacumed®, FL Medical, Torreglia, Italy) using a 20 gauge needle in a closed evacuated system (FL Medical, Torreglia, Italy). To eliminate any possible interference due to either the contact phase or tissue factor, approximately 2 mL of blood were preliminarily collected in a discard tube without additives (Vacumed®, FL Medical, Torreglia, Italy). The blood collection procedure was appropriately standardized in each phase, as already reported [20, 21], particularly regarding the sample processing, centrifugation and serum/plasma separation.

All the samples were assayed in a single analytical run in the same instrument according to the manufacturer's specifications and using proprietary reagents. The panel of tests that were performed and the instruments used by the Center of Laboratory Medicine of the Vilnius University Hospital Santaros Klinikos are shown in Table 2.

The instruments were calibrated against appropriate proprietary reference standard materials and verified with independent third-party control materials (Liquid Assayed Multiqual® Level 1 for routine biochemistry tests (Bio-Rad, California, USA) and Multichem IA Plus® Level 1 for immunochemistry assays (Technopath Clinical Diagnostics, Ballina, Ireland)) [22].

The evaluation of the within-run precision by the internal quality control of the instruments used in this study showed low coefficients of variation (Table 2).

Table 2 Results of within-run precision by the internal quality control used on the instruments

Part I: Clinical chemistry				
Instrument	**Test**	**Method**	**IQC assigned value**	**CVa (%)**
Arhitect ci8200, Abbott	Glc	enzymatic, Hexokinase / G-6-PDH, UV	3.13 mmol/L	1.2
	Cortisol	CMIA	127 nmol/L	7.4
	Ins	CMIA	17.1 pmol/L	6.7
	C peptide	CMIA	39.1 nmol/L	4.6
	CHOL	enzymatic, cholesterol oxidase / cholesterol esterase	2.90 mmol/L	0.5
	HDL	accelerator selective detergent, cholesterol oxidase / cholesterol esterase	0.74 mmol/L	2.5
	TG	enzymatic, glycerol phosphate oxidase	0.91 mmol/L	1.4
	TP	biuret	38.1 g/L	0.5
	Alb	bromocresol green, colorimetric	0.36 mmol/L	0.4
	UA	enzymatic, uricase	0.21 mol/L	6.8
	Urea	UV, urease	2.46 mmol/L	4.0
	CREA	enzimatic	0.08 mmol/L	4.1
	CRP	latex immunoturbidimetric	8.10 nmol/L	2.6
	ALP	p-nitrophenyl phosphate	34.2 U/L	2.8
	AMY-P	enzymatic, colorimetric	23.3 U/L	4.3
	AMY	CNPG3 substrate	45.4 U/L	1.6

	AST	IFCC, NADH, with P5P	40.6 U/L	7.3
	ALT	IFCC, NADH, with P5P	24.5 U/L	1.2
	GGT	L-Gamma-glutamyl-3-carboxy-4-nitroanilide substrate	29.5 U/L	3.2
	LD	IFCC, UV lactate-pyruvate	116 U/L	0.6
	Lip	quinone dye	20.6 U/L	8.2
	CK	N-acetyl-L-cysteine, NAC	83.5 U/L	4.8
	TBIL	diazonium salt	11.8 µmol/L	1.0
	DBIL	diazo reaction	5.82 µmol/L	7.8
	Phos	UV, phosphomolybdate	0.61 mmol/L	0.3
	Ca	arsenazo III, colorimetric	1.54 mmol/L	0.4
	Mg	enzymatic, isocitrate dehydrogenase	0.77 mmol/L	4.9
	Fe	ferene, colorimetric	12.2 µmol/L	4.0
	Na	ion-selective electrode, indirect	117 mmol/L	0.9
	K	ion-selective electrode, indirect	2.59 mmol/L	1.1
	Cl	ion-selective electrode, indirect	78.9 mmol/L	1.0
	TSH	CMIA	0.048 µIU/L	11.0
	fT4	CMIA	11.2 pmol/L	3.3
Part II: Hematology				
XN-1000, Sysmex	RBC	impedance	4.50 10^{12}/L	8.0
	Hct	calculated	0.47 L/L	0.3
	Hb	photometric	155 g/L	0.7
	MCV	calculated	88.5 fL	0.1

	RDW	calculated	15.2 %	0.5
	WBC	flourescent flow citometry	9.10 10^9/L	1.5
	Lympho	flourescent flow citometry	2.4 10^9/L	3.7
	Mono	flourescent flow citometry	0.8 10^9/L	4.1
	Neu	flourescent flow citometry	5.3 10^9/L	2.7
	Eos	flourescent flow citometry	0.6 10^9/L	17.3
	Baso	flourescent flow citometry	0.33 10^9/L	11.8
	IG	flourescent flow citometry	0.75 10^9/L	16.1
	Plt	impedance	245 10^9/L	2.2
	PDW	calculated	9.2 %	2.8
	MPV	calculated	9.8 fL	0.8
Part III: Coagulation				
STA Compact Max, Diagnostica Stago	PT	coagulometric, Owren	14.7 s	6.5
	APTT	coagulometric, silica	35.5 s	7.7
	Fbg	coagulometric, Clauss	2.9 g/L	11.1
	PC	chromogenic, Agkistrodon c. contortrix venom	96 %	7.3
	PS	latex immunoturbidimetric	62 %	12.6

IQC – internal quality control; CVa – analytical coefficient of variation; Glc – glucose; Ins – insulin; CHOL – cholesterol; HDL – high density lipoprotein; TG – triglycerides; TP – total protein; Alb – albumin; UA – uric acid; CREA – creatinine; CRP – C reactive protein; ALP – alkaline phosphatase; AMY-P – pancreatic amylase; AMY – amylase; AST – aspartate aminotransferase; ALT – alanine aminotransferase; GGT – gamma glutamyl transferase; LD – lactate dehydrogenase; Lip – lipase; CK – creatine kinase; TBIL – total bilirubin; DBIL – direct bilirubin; Phos – phosphate; Ca – calcium; Mg – magnesium; Fe – iron; Na – sodium; K – potassium; Cl – chloride; TSH – thyroid stimulating hormone; fT4 – free thyroxin; RBC – red blood cells; Hct – haematocrit; Hb – haemoglobin; MCV – mean cell volume; RDW – red cell distribution width; WBC – white blood cells; Lympho – lymphocytes; Mono – monocytes; Neu – neutrophils; Eos – eosinophils; Baso – basophils; IG – immature granulocytes; Plt – platelets; PDW – platelet distribution width; MPV – mean platelet volume; PT – prothrombin time; APTT – activated partial thromboplastin time; Fbg – fibrinogen; PC – protein C; PS – protein S; UV – ultraviolet; CMIA – chemiluminescent microparticle immunoassay; NADPH - nicotinamide adenine dinucleotide phosphate; NAC – N-acetyl cysteine; IFCC – international federation of clinical chemistry and laboratory medicine.

For assessing the statistical differences between samples, the Wilcoxon ranked-pairs test was used in agreement with Simundic's [23] recommendations regarding sample size (i.e. less than 30), with a licensed statistical software (GraphPad Prism® version 5.01, La Jolla, CA, USA). The level of statistical significance was set at $p < 0.05$.

Presently there is a lack of harmonization on the preanalytical methodology for evaluating a single source of laboratory variability (e.g., impact of chewing gum). Researchers use different statistical tools to estimate bias and clinical significance: (i) bland-Altman analysis [24]; (ii) percentage mean difference [25] – which represents the difference between baseline and treatment values divided by the treatment value – the method we chose; and iii) Passing-Bablok regression with 95% confidence intervals [26].

To calculate the percentage mean difference, we decided to use the following formula:

mean % difference =
[(× h after chewing sugar-free gum − basal)/× h after chewing sugar-free gum] × 100%.

Using it we could avoid the possible "false positive source of variability" since it is more sensitive than:

mean % difference =
[(basal – x h after chewing sugar-free gum) / basal] × 100%.

In this way, only strong sources of preanalytical variability can be identified.

Finally, the mean % differences between blood samples taken 1, 2 and 4 hours after chewing sugar-free gum, were compared with the desirable specification for imprecision (DSI) derived from biologic variation [27]. We used DSI as criterium of acceptable level of interference by chewing gum in the laboratory tests; then interferograms were provided for each laboratory parameters with significant difference between h after chewing sugar-free gum and basal.

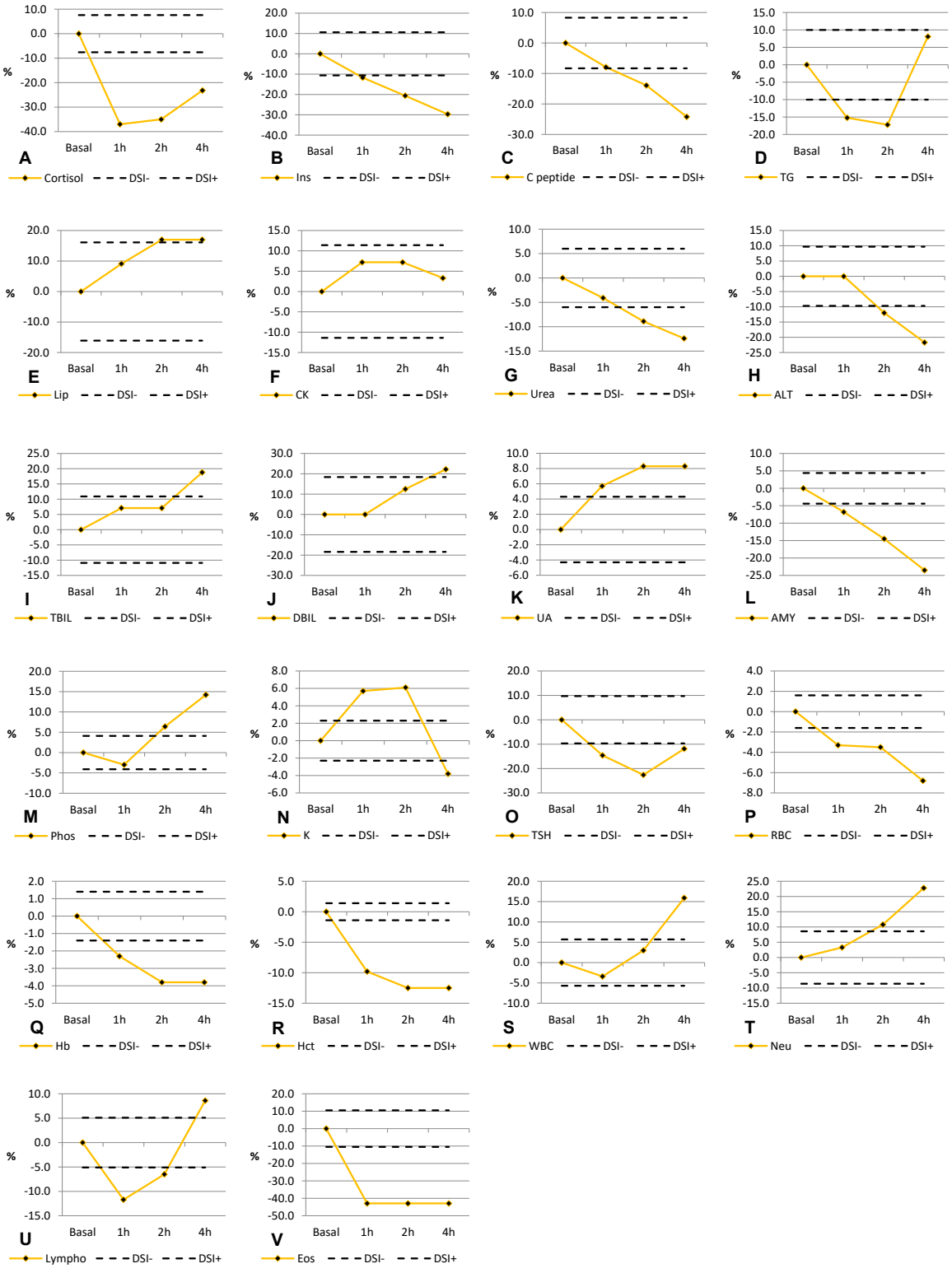
Briefly, in our study design, each volunteer was devised to be her/his own control (i.e. the results from 1, 2 and 4 hours after chewing sugar-free gum were compared with basal-results of the same individual). Indeed, this kind of study design – a case-crossover study – is most suitable for outcomes where the induction time is short [28], like our evaluation of impact of chewing gum on laboratory test results. In fact, in a case-crossover study, only cases showing discordant exposure status in the case/control window-of-time contribute to the effect and thus to the measure estimation. Because cases and controls are the same individuals, the problem of between-person confounders—that anyway exists with a control group—being constant for the characteristics, do not occur [29]. Therefore, our design minimizes the variability that could jeopardize the preanalytical evaluation.

RESULTS

The results of the laboratory tests are presented as median [interquartile range] in Table 3.

Among all the results, statistically significant differences between basal and x h after chewing sugar-free gum were observed for the following parameters: cortisol, insulin, C peptide, triglycerides, uric acid, urea, amylase, alanine aminotransferase, lipase, creatine kinase, total bilirubin, direct bilirubin, phosphate, iron, potassium, thyroid stimulating hormone, red blood cell count, hematocrit, hemoglobin, mean cell volume, red cell distribution width, white blood cell count, lymphocytes, neutrophils, and eosinophils; whereas, coagulation tests were not impacted by chewing sugar-free gum (Figure 1).

Figure 1 **Interferograms**



Legend follows on next page.

Legend of Figure 1:
A, cortisol; B, Ins – insulin; C, C-peptide; D, TG – triglycerides; E, Lip – lipase; F, CK – creatine kinase; G, urea; H, ALT – alanine aminotransferase; I, TBIL – total bilirubin; J, DBIL – direct bilirubin; K, UA – uric acid; L, MY – amylase; M, Phos – phosphate; N, K – potassium; O, TSH – thyroid stimulating hormone; P, RBC – red blood cells; Q, Hb – haemoglobin; R, Hct – haematocrit; S, WBC – white blood cells; T, Neu – neutrophils; U, Lympho – lymphocytes; and V, Eos – eosinophils
Hours after the chewing the gum (x-axis) are plotted against bias values (y-axis). Solid line – bias. Dashed lines - acceptable criteria based on desirable specification for imprecision (DSI) derived from biologic variation.

Table 3 **Laboratory test results variation after chewing sugar-free gum**

Part I: Clinical chemistry					
Test	**Unit**	**Basal**	**1h**	**2h**	**4h**
Glc	mmol/L	4.61 [4.30 - 4.78]	4.60 [4.31 - 4.77] 0.758	4.51 [4.43 - 4.84] 0.646	4.49 [4.32 - 4.71] 0.268
Cortisol	nmol/L	290 [219 - 383]	212 [149 - 279] **0.002**	215 [183 - 290] **0.003**	235 [172 - 292] **0.018**
Ins	pmol/L	41.0 [30.6 - 67.9]	36.7 [24.9 - 51.8] **0.002**	34.0 [24.5 - 55.1] **0.004**	31.6 [22.0 - 42.9] **0.001**
C peptide	nmol/L	0.41 [0.36 - 0.62]	0.38 [0.33 - 0.56] **0.003**	0.36 [0.30 - 0.53] **0.002**	0.33 [0.26 - 0.46] **<0.001**
CHOL	mmol/L	4.68 [4.34 - 5.58]	5.59 [4.32 - 4.46] 0.641	5.60 [4.24 - 5.48] 0.673	4.68 [4.37 - 5.48] 0.962
HDL	mmol/L	1.59 [1.38 - 1.96]	1.58 [1.35 - 1.92] 0.972	1.59 [1.33 - 1.90] 0.822	1.57 [1.32 - 1.88] 0.511
TG	mmol/L	0.68 [0.54 - 0.83]	0.59 [0.48 - 0.87] **0.017**	0.58 [0.50 - 0.86] **0.005**	0.74 [0.57 - 0.88] **0.001**
TP	g/L	76.3 [71.8 - 77.9]	75.4 [72.0 - 77.7] 0.788	75.3 [71.8 - 78.1] 0.714	75.5 [72.8 - 80.4] 0.754

Alb	g/L	46.3 [44.5 - 48.0]	46.5 [44.7 - 47.3] 0.651	46.4 [43.6 - 49.8] 0.642	46.5 [45.7 - 48.0] 0.653
UA	mmol/L	0.33 [0.25 - 0.39]	0.35 [0.27 - 0.41] **0.009**	0.36 [0.27 - 0.42] **0.005**	0.36 [0.27 - 0.42] **0.002**
Urea	mmol/L	4.68 [3.75 - 5.79]	4.50 [3.70 - 5.64] **0.001**	4.30 [3.66 - 5.51] **0.001**	4.16 [3.68 - 5.46] **0.001**
CREA	mmol/L	0.08 [0.06 - 0.09]	0.08 [0.07 - 0.09] 0.876	0.08 [0.07 - 0.09] 0.829	0.08 [0.07 - 0.09] 0.798
CRP	nmol/L	4.76 [2.86 - 5.81]	4.67 [2.76 - 6.19] 0.729	4.86 [2.76 - 6.67] 0.851	4.76 [2.86 - 6.19] 0.892
ALP	U/L	56 [48 - 66]	57 [47 - 62] 0.876	57 [49 - 62] 0.841	57 [47 - 61] 0.865
AMY-P	U/L	27 [21 - 35]	27 [21 - 30] 0.823	27 [20 - 31] 0.888	27 [21 - 31] 0.891
AMY	U/L	63 [55 - 80]	59 [49 - 78] **0.002**	55 [45 - 78] **0.001**	51 [40 - 68] **0.001**
AST	U/L	23 [17 - 29]	22 [20 - 28] 0.751	23 [19 - 29] 0.704	22 [19 - 30] 0.781
ALT	U/L	28 [17 - 38]	28 [16 - 37] 0.979	25 [16 - 32] **0.021**	23 [15 - 30] **0.002**
GGT	U/L	17 [10 - 22]	17 [10 - 23] 0.978	17 [10 - 24] 0.813	17 [10 - 24] 0.838

LD	U/L	185 [161 - 200]	187 [163 - 201] 0.439	186 [160 - 203] 0.483	186 [160 - 199] 0.481
Lip	U/L	20 [17 - 23]	22 [16 - 22] **0.002**	24 [18 - 26] **0.001**	24 [18 - 27] **0.001**
CK	U/L	116 [71 - 130]	125 [79 - 145] **0.002**	125 [78 - 146] **0.001**	120 [80 -138] **0.021**
TBIL	µmol/L	11.1 [8.55 - 12.3]	12.0 [8.38 - 13.7] 0.091	12.0 [8.21 - 14.0] 0.083	13.7 [10.6 - 16.2] **0.002**
DBIL	µmol/L	4.79 [3.59 - 5.64]	4.79 [3.76 - 5.64] 0.784	5.47 [4.10 - 6.16] **0.002**	6.17 [4.28 - 6.84] **0.001**
Phos	mmol/L	1.03 [0.94 - 1.23]	1.00 [0.97 - 1.17] **0.035**	1.10 [1.00 - 1.21] **0.036**	1.20 [1.10 - 1.23] **0.002**
Ca	mmol/L	2.43 [2.42 - 2.46]	2.44 [2.40 - 2.47] 0.829	2.44 [2.39 - 2.47] 0.794	2.44 [2.37 - 2.48] 0.817
Mg	mmol/L	0.83 [0.79 - 0.86]	0.83 [0.79 - 0.84] 0.912	0.83 [0.79 - 0.84] 0.897	0.83 [0.80 - 0.84] 0.849
Fe	µmol/L	18.1 [12.4 - 23.3]	18.3 [12.2 - 23.1] **0.046**	18.3 [12.5 - 23.5] **0.047**	19.5 [14.7 - 23.3] **0.005**
Na	mmol/L	140 [139 - 141]	140 [140 - 141] 0.887	140 [138 - 140] 0.732	141 [140 - 143] 0.481
K	mmol/L	4.15 [3.91 - 4.22]	4.40 [4.21 - 4.48] **0.003**	4.42 [4.20 - 4.40] **0.004**	4.00 [3.89 - 4.18] **0.023**

Cl	mmol/L	106 [104 - 106]	106 [104 - 107] 0.910	106 [105 - 107] 0.931	106 [104 - 107] 0.914
TSH	µIU/L	1.41 [0.94 - 2.10]	1.23 [0.84 - 1.81] **<0.001**	1.15 [0.93 - 1.71] **<0.001**	1.26 [0.95 - 1.85] **<0.001**
fT4	pmol/L	12.9 [11.7 - 13.5]	12.9 [12.0 - 13.8] 0.752	12.7 [11.7 - 13.6] 0.801	12.7 [11.9 - 13.9] 0.842
Part II: Hematology					
RBC	10^{12}/L	4.70 [4.33 - 5.20]	4.55 [4.29 - 5.03] **0.001**	4.54 [4.20 - 5.02] **0.001**	4.40 [4.25 - 4.90] **0.001**
Hct	L/L	0.45 [0.40 - 0.46]	0.41 [0.37 - 0.46] **0.001**	0.40 [0.38 - 0.45] **0.001**	0.40 [0.36 - 0.45] **0.001**
Hb	g/L	135 [126 - 149]	132 [122 - 147] **0.001**	130 [122 - 148] **0.001**	130 [121 - 147] **0.001**
MCV	fL	90.8 [87.3 - 92.0]	90.0 [86.8 - 91.4] **0.001**	89.1 [86.0 - 90.6] **0.001**	87.9 [85.0 - 90.1] **0.001**
RDW	%	44.0 [41.0 - 46.0]	43.0 [40.2 - 45.9] **0.001**	42.1 [40.0 - 43.9] **<0.001**	41.0 [39.3 - 44.2] **<0.001**
WBC	10^{9}/L	5.20 [4.01 - 5.74]	5.03 [4.18 - 5.72] 0.548	5.36 [4.42 - 6.01] 0.062	6.18 [5.11 - 6.98] **0.007**
Lympho	10^{9}/L	1.81 [1.40 - 1.93]	1.62 [1.51 - 1.80] **<0.001**	1.70 [1.52 - 2.03] **<0.001**	1.98 [1.78 - 2.31] **<0.001**

Mono	10^9/L	0.41 [0.28 - 0.54]	0.41 [0.29 - 0.50] 0.618	0.38 [0.29 - 0.47] 0.714	0.45 [0.31 - 0.53] 0.839
Neu	10^9/L	2.64 [1.98 - 3.10]	2.73 [2.20 - 3.32] **0.041**	2.96 [2.44 - 3.41] **0.031**	3.42 [2.63 - 4.10] **0.001**
Eos	10^9/L	0.10 [0.06 - 0.14]	0.07 [0.05 - 0.12] **0.001**	0.07 [0.05 - 0.10] **0.001**	0.07 [0.04 - 0.10] **0.001**
Baso	10^9/L	0.04 [0.02 - 0.04]	0.04 [0.02 - 0.04] 0.792	0.04 [0.02 - 0.04] 0.859	0.04 [0.03 - 0.04] 0.817
IG	10^9/L	0.01 [0.00 - 0.01]	0.01 [0.01 - 0.01] 0.759	0.01 [0.00 - 0.02] 0.893	0.01 [0.01 - 0.02] 0.848
Plt	10^9/L	228 [204 - 269]	226 [204 - 268] 0.818	225 [204 - 267] 0.836	229 [206 - 269] 0.891
PDW	%	11.2 [10.0 - 12.0]	11.5 [9.74 - 12.1] 0.737	11.7 [9.98 - 12.4] 0.761	11.4 [10.0 - 12.1] 0.729
MPV	fL	10.0 [9.28 - 10.3]	10.0 [9.35 - 10.2] 0.751	10.1 [9.50 - 10.1] 0.849	10.1 [9.51 - 10.2] 0.772
Part III: Coagulation					
PT	s	13.0 [12.6 - 13.2]	13.0 [12.7 - 13.3] 0.618	13.1 [12.8 - 13.4] 0.684	13.1 [12.7 - 13. 3] 0.679
APTT	s	33.1 [32.2 - 36.0]	33.2 [32.1 - 35.9] 0.841	33.0 [31.6 - 36.5] 0.973	33.1 [32.0 - 35.2] 0.897

Fbg	g/L	2.94 [2.53 - 3.12]	2.88 [2.50 - 3.15] 0.529	2.89 [2.41 - 3.22] 0.671	2.89 [2.42 - 3.16] 0.541
PC	% activity	102 [89.3 - 120]	100 [89.7 - 119] 0.638	100 [89.9 - 119] 0.699	101 [90.3 - 117] 0.594
PS	% activity	88.3 [75.5 - 111]	87.8 [75.2 - 111] 0.482	88.2 [76.2 - 112] 0.875	88.7 [76.1 - 111] 0.897

Results are presented as median [interquartile range]. Statistically significant differences (P < 0.05) are presented in bold. Glc – glucose; Ins – insulin; CHOL – cholesterol; HDL – high density lipoprotein; TG – triglycerides; TP – total protein; Alb – albumin; UA – uric acid; CREA – creatinine; CRP – C reactive protein; ALP – alkaline phosphatase; AMY-P – pancreatic amylase; AMY – amylase; AST – aspartate aminotransferase; ALT – alanine aminotransferase; GGT – gamma glutamyl transferase; LD – lactate dehydrogenase; Lip – lipase; CK – creatine kinase; TBIL – total bilirubin; DBIL – direct bilirubin; Phos – phosphate; Ca – calcium; Mg – magnesium; Fe – iron; Na – sodium; K – potassium; Cl – chloride; TSH – thyroid stimulating hormone; fT4 – free thyroxin; RBC – red blood cells; Hct – haematocrit; Hb – haemoglobin; MCV – mean cell volume; RDW – red cell distribution width; WBC – white blood cells; Lympho – lymphocytes; Mono – monocytes; Neu – neutrophils; Eos – eosinophils; Baso – basophils; IG – immature granulocytes; Plt – platelets; PDW – platelet distribution width; MPV – mean platelet volume; PT – prothrombin time; APTT – activated partial thromboplastin time; Fbg – fibrinogen; PC – protein C; PS – protein S.

DISCUSSION

Our primary hypothesis was that chewing gum – even sugar free – could impact the glucose metabolism. However, the results of glucose, cortisol, insulin and C peptide levels properly evidenced that chewing gum lacks influence on glucose results (Table 3); the variability observed in cortisol, insulin and C peptide (Table 3, Figure 1a, 1b, 1c) could mirror cortisol circadian cycle [30] (since the first blood collection was at 8 am), and life cycle of these hormones [31]. Studies have shown that the insulin half-life varies between 4.3 and 9.8 min, whereas for C peptide the half-life could vary between 11.1 and 33.5 min [32-35]. Therefore, our insulin and C peptide results could mirror the prolonged fasting.

The chewing gum producer declares the following chemical constituents of the product on the pack label: a) sweeteners: E967 – xylitol, E420 – sorbitol, E951 – aspartame, E421 – mannitol, E950 – acesulfame potassium, and E955 – sucralose; b) moisture additive: E422 – glycerol; c) thickener: E414 – gum Arabic; d) emulsifier: soy lecithin; e) food colouring: E171 – titanium dioxide; f) filler: E903 – carnauba wax; g) antioxidant: E320 – butylated hydroxyanisole. However, the chewing gum producer do not specify the quantities of mentioned constituents, furthermore, they do not specify the base of the gum (i.e., it is not declared on the gum package). Probably it is a mixture of resins, elastomers, fillers, and plasticizers [2].

Regarding lipids profile, total cholesterol and HDL cholesterol were not affected neither by prolonged fasting time [8] nor chewing gum (Table 3). However, the decrease in triglycerides one and two hours after chewing gum can mirror the gluconeogenesis and beta oxidation process, since volunteers had fasted for 13 and

14 hours prior to blood collections, respectively. However, four hours after chewing gum the triglycerides concentration increased and returned almost up to the basal levels (Table 3, Figure 1d). Since glycerol is the moisture additive declared by the chewing gum producer (Table 1), it could be absorbed and provide positive bias in our enzymatic, glycerol phosphate oxidase method for triglyceride test. Moreover, the methodology of *quinone dye* is used on Architect to determine lipase activity. Briefly, lipase acts on a natural substrate, 1,2-diglyceride, to liberate 2-monoglyceride. This is hydrolyzed by monoglyceride lipase into glycerol and free fatty acid. Glycerol kinase acts on glycerol to form glycerol-3-phosphate which is in turn acted on by glycerol-3-phosphate oxidase to generate hydrogen peroxide. Peroxidase converts the hydrogen peroxide, 4-aminoantipyrine, and N-ethyl-N-(2-hydroxy-3-sulfopropyl)-m-toluidine (TOOS) into a quinone dye. Thus, the rate of formation of the dye is measured as an increase in absorbance at 548 nm and is proportional to the lipase activity in the sample. Therefore, the glycerol from sugar-free chewing gum could potentially jeopardize lipase results (Table 3, Figure 1e).

The oxidation of NADPH by the radical of butylated hydroxyanisole (BHA, present in the chewing gum) was previously demonstrated [36, 37]. The short decryptions of the laboratory methods below could explain the variability observed in CK, urea, and ALT (Table 3, Figure 1f, 1g, 1h).

- CK: in N-acetyl-L-cysteine (NAC) methodology, the creatine kinase, present in the sample, catalyzes the transfer of a high energy phosphate group from creatine phosphate to ADP. The ATP produced in this reaction is subsequently used to phosphorylate glucose to produce glucose-6-phosphate (G-6-P) in the presence of hexokinase. G-6-P is then oxidized by glucose-6-phosphate dehydrogenase (G-6-PDH) with the concomitant reduction of nicotinamide adenine dinucleotide phosphate (NADP) to nicotinamide adenine dinucleotide phosphate reduced (NADPH). The rate of formation of NADPH is monitored at 340 nm and is proportional to the activity of CK in the sample. These reactions occur in the presence of N-acetyl-L-cysteine (NAC) which is present as an enzyme reactivator;
- Urea: in urease methodology, two moles of NADH are oxidized for each mole of urea present. The initial rate of decrease in absorbance at 340 nm is proportional to the urea concentration in the sample;
- ALT: in IFCC methodology, the pyruvate in the presence of NADH and lactate dehydrogenase is reduced to L-lactate. In this reaction NADH is oxidized to NAD. The reaction is monitored by measuring the rate of decrease in absorbance at 340 nm due to the oxidation of NADH to NAD.

Moreover, Vandghanooni et al. experimentally demonstrated that slight concentration of butylated hydroxyanisole – lower than expected from human exposure to antioxidants in food products, i.e. chewing gum – inhibits the growth rate of cells by inducing apoptosis via chromatin and DNA fragmentation [38]. Furthermore, the xylitol in the sugar-free chewing gum could be responsible for the increase observed in bilirubin, and uric acid [39, 40], and decrease in amylase [41] (Table 3, Figure 1i, 1j, 1k, 1l).

Similar results on phosphate, potassium and TSH (Table 3, Figure 1m, 1n, 1o) were demonstrated by Bajaña et al. [5] on volunteers after breakfast. Therefore, a question remains partially unanswered “Is the chewing gum able to activate digestive pathway?”. Regarding Smith et al. [4] chewing gum is, at least partially, able to stimulate the initial phases of gastric secretion that involve essentially water and ions’ movements. Our results seem to support the relative effect of haemoconcentration as deduced by red blood cell count, haemoglobin and

hematocrit decrease, an effect that appears reversible with time according to the physiological process of digestion (Table 3, Figure 1p, 1q, 1r).

Moreover, fake feeding-induced gastric secretion due to chewing gum is probably responsible for the white blood cell count and neutrophils increase, with lymphocytes and eosinophils decrease observed in our study (Table 3, Figure 1s, 1t, 1u, 1v) as reported by Koscielniak et al. after a normal meal [42]. On the contrary the sugar-free chewing gum does not have impact on routine coagulation tests (Table 3).

In conclusion, based on the variability evidenced above, we recommend instructing the patients to avoid the use of chewing gum before blood collection for laboratory tests.

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