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Frailty syndrome, biomarkers and environmental factors – A pilot study

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ABSTRACT

Frailty is an age-related syndrome expected to increase over the next decades. This syndrome has been identified to be the most common condition leading to disability, institutionalisation and death in the elderly. The aim of this pilot study is to investigate a possible link between frailty status, biomarkers and environmental exposures. A group of 71 older adults (≥65 years old) was engaged in this study. The study population was classified as 45.1% robust, 45.1% pre-frail and 9.8% frail. A significant higher prevalence of second-hand smokers was found in the pre-frail group when compared to robust. Furthermore, a higher prevalence of robust individuals was found among those consuming home-produced vegetables and water from well/springs. Significant differences were found between data collected in a lifetime exposure questionnaire (LTEQ) and the levels of genotoxicity endpoints and the mercury levels analysed regarding some exposure-related parameters, namely, smoking habits, intake of home-produced vegetables and the use of pesticides in agriculture. Understanding if the way we live(d) or worked can impact the way we age are important questions to be explored. Data obtained in this pilot study encourage further studies on this matter, exploring the role of exposures history and its impact on health.

1. Introduction

The world is ageing rapidly, group aged 60 years or over is growing at a rate of about 3% per year (United Nations, 2017). This demographic shift has clear socioeconomic implications, offering enormous opportunities and great challenges for all societies. These and other challenges must be addressed by anticipating coming demographic trends and incorporating that information into policies and planning.

Recent evidence advocates that healthy aging may be possible with morbidity compressed to the later years of life (Cohen and Gerber, 2017). Hence, susceptibility associated with advancing age may result not from a direct age effect but rather from age acting as an imperfect surrogate for health status (Eckel et al., 2012). Health status in older adults (≥65 years old) is complex and multidimensional. One metric is frailty, a state of increased vulnerability to stressors, characterised by decreased physical and mental functioning and an increased risk for poor health outcomes (Fries et al., 2011; Villacampa-Fernandez et al., 2017). The progressive accumulation of health deficits increases the risk of disease onset and leads to a cycle of events (i.e. fatigue, weight loss, undernutrition, sarcopenia, diminished strength) that perpetuate frailty. With the addition of new stressors, the cycle of frailty progresses leading to disability, hospitalisation, institutionalisation and ultimately death (Ruiz et al., 2012). Nowadays, the estimated prevalence of frailty syndrome in older adults is around 15%, although in some settings proportions up to 59% have been reported (Collard et al., 2012; Santos-Eggimann et al., 2009).

Recognised as a medical syndrome, frailty diagnosis is commonly based on five phenotypical criteria established by Fried et al. (2001).

Abbreviations: %TDNA, percentage of DNA in the comet tail; ADL, basic activities of daily living; B-Hg, mercury blood levels; IADL, instrumental activities of daily living; JEM, job-exposure matrix; LTEQ, lifetime exposure questionnaire; MNA-SF, Mini Nutritional Assessment short-form; PI, propidium iodide; PPE, personal protective equipment

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Although this model remains the most solid in clinical practice and research, some components of this phenotype are more relevant than others, hence different models based on different features and scores emerged to overcome these limitations. Despite the model, their ability to predict and recognise the evolution between robust or pre-frail to frail is still limited (Saedi et al., 2019). Nevertheless, frailty phenotype has been considered more suitable for the immediate identification of non-disabled older adults who are at increased risk for negative events, such as non-institutionalised community-dwellers (Chen et al., 2015). Given the serious consequences of frailty and its high frequency, research is being focused on identifying mechanistic pathways and preventable risk factors (Clegg et al., 2014; Fairhall et al., 2012).

While generally recognised to have a biological basis, no particular biological trait has been consistently associated with frailty status so far (Sanchez-Flores et al., 2017). Genomic instability has been considered one of the hallmarks of aging and age-related diseases (Lopez-Otin et al., 2013), as well as variations in endocrine and immune systems (Clegg and Hassan-Smith, 2018; Leng et al., 2011). Some positive associations were found at immunological (Marcos-Perez et al., 2017, 2018) and endocrine level (Collerton et al., 2012; Fulop et al., 2015) namely with regard to circulating markers of systemic inflammatory response (C-reactive protein (CRP), IL-6, tumour necrosis factor-alpha) (Marcos-Perez et al., 2018) and hormonal changes (decreasing of testosterone and Vitamin D levels, (Saedi et al., 2019) and increasing cortisol levels (Marcos-Perez et al., 2019)). Variations of oxidative stress biomarkers levels have also been correlated with frailty status (Sanchez-Flores et al., 2017); for genomic instability positive findings were reported on recent studies (Sanchez-Flores et al., 2018; Valdiglesias et al., 2019). Although studies on this field are still limited, the link established between molecular biomarkers and age-related disorders (Partridge et al., 2018) encourage deeper investigations.

Some studies have found a correlation between exposure to pollutants (e.g. particulate matter, water contaminants, metals, tobacco smoke) and the development of age-related diseases, including frailty syndrome (Eckel et al., 2012; García-Esquinas et al., 2015a). Study of environmental health in the elderly has a great deal in common with the study of children's health because it highlights the dynamic interaction of timing, functions, and environmental exposures. Furthermore, for some ubiquitous contaminants (e.g. metals, pesticides) this increased risk may be related to the accumulation in the human body over lifetime, resulting in chronic endogenous exposure of target tissues (García-Esquinas et al., 2015a). For instance, the contamination by mercury is a concerning public health problem in Portugal and other Mediterranean-style diet countries, since fish is an important component of the everyday diet (Nunes et al., 2014). Because of the variability of health effects resulting from exposure to environmental agents in the elderly, individual functions should be viewed in the context of genetics, health status, nutrition, occupation, and socioeconomic status. Since frailty syndrome is an aged-related disorder with undetermined causal effects, environmental exposures should also be considered when studying the potential risk factors of the syndrome.

The complex underlying pathophysiology make the development of biomarkers for this condition extremely challenging. The early knowledge of the frailty state is crucial to prevent the progressive decline and even more considering that frailty is potentially reversible at a certain stage.

The aim of this study was to identify early biomarkers of DNA damage potentially linked to frailty status as well as to screen risk factors related to syndrome aetiology. The levels of primary DNA damage and oxidative DNA were measured by comet assay, as well as H2AX phosphorylation by γH2AX assay. To explore the potential environmental-frailty link a lifetime exposure questionnaire (LTEQ) was firstly constructed and applied. The levels of blood mercury were also evaluated. To our knowledge, this is the first field-study investigating the influence of environmental exposures on frailty syndrome status, and also constructing and applying a LTEQ to older adults.

2. Materials and methods

2.1. Study population

A group of 71 community dwellers aged 65 years old or more were recruited from the metropolitan region of Porto (Northern Portugal). All subjects were fully informed on the principal aims of the study, nature of participation, risks and benefits. Before being enrolled in the study each subject was asked to sign the informed consent form. The participation in the study was voluntary (no financial compensation was offered) and stopped if requested at any point. The study was approved by the Ethics Committee of the Instituto de Saúde Pública da Universidade do Porto (ISPUP) and authorized by the National Commission for Data Protection (CNPD-Comissão Nacional de Proteção de Dados) (Authorization no 5446/2018). Eligible for inclusion in the study were subjects above 65 years old and living in the metropolitan region of Porto. The exclusion criteria includes: a) severe dementia and/or cognitive impairment, b) lack of ability to communicate, c) severe impairment of sight and hearing and d) receiving palliative care. A comprehensive geriatric evaluation was carried out comprising socio-clinical information (medical, lifestyle and demographic data) including diet features assessed by the Mini Nutritional Assessment short-form (MNA-SF) (Kaiser et al., 2009). Furthermore, standard scales were also applied for cognitive dysfunction and for functional status valuation, such as basic activities of daily living (ADL) and instrumental activities of daily living (IADL). Cognitive dysfunction was assessed via the Mini-Mental State Examination (Folstein et al., 1975; Guerreiro et al., 1994), ADL by the Katz Index (Katz et al., 1963) and IADL by the Lawton-Brody scale (Lawton and Brody, 1969).

2.2. Frailty status

Subject's frailty status was assessed according to Fried et al. (2001) with some minor modifications. Participants were evaluated on five phenotypical criteria: i) unintentional weight loss (self-reported), ii) self-reported exhaustion, iii) low physical activity, iv) slow walking time, and v) weakness of grip strength (measured by a JAMAR®Hydraulic Hand Dynamometer). Following the cut-offs established by the model, each criterion was punctuated with "zero" if absent or with "one" if present. The final sum of the five criteria sets the frailty status: frail, if scoring in three or more criteria, pre-frail, if scoring only in one or two criteria, or robust, with zero criterion scored. For cut-off application weight was measured using a SECA® 761 flat mechanic scale to the nearest 0.5 kg (SECA GMBH & Co. Kg., Hamburg, Germany) and height recorded to the nearest millimeter using a portable stadiometer SECA® 213 (SECA GMBH & Co. Kg., Hamburg, Germany). The low energy expenditure parameter was assessed by the adapted Portuguese version of the Spanish Minnesota Leisure Time Activities short version questionnaire (VREM) (Ruiz Comellas et al., 2012) converting weekly tasks to equivalent kilocalories of expenditure. VREM presents some advantages compared with the version used by Fried et al. (2001) namely its specificity for older adults' population, short time required and ease of application and interpretation.

2.3. Lifetime exposure questionnaire (LTQE)

To investigate the potential environmental-frailty link a LTEQ was constructed with inquiries regarding current/past environmental exposure and occupational history data. LTEQ was based on a monography by the Agency for Toxic Substances and Disease Registry (ATSDR, 2010). The LTEQ has 22 questions divided in 4 main areas/themes: a) current environmental exposure, with questions on residence area, household proximity to traffic and other sources of local pollution, house conditions and equipment use; b) food and water source; c) occupational exposure history, with questions regarding activities and tasks performed, workplaces, exposure to known hazards and work

conditions, use of personal protective equipment (PPE); and, d) general question regarding previous households conditions and residence location. Additionally, recreational past exposure linked to farming was also evaluated taking in account the socio and cultural history of the elderly population living in metropolitan region of Porto. The presence of sources of local pollution near the household, namely farms (agricultural production), livestock farming, slaughterhouses, landfills, mines, industries and/or traffic zones, represents the possibility of exposure to different air and water contaminants. As stated herein, the source of fruit and vegetables (commercial or private farmhouse) and water for consumption (public, bottled, well or spring water source) were also taken in account. Consumption of bottled water or running water was considered a non-risk exposure situation, but using water from wells or spring water was considered a potential risk of exposure to contaminants. Commercial origin represents a controlled and safer origin, and for this reason, in this study it was defined as non-exposure source. In contrast, farmhouse-based products were considered a source of possible human exposure to contaminants. Previous farming activities were also evaluated with regard to the use of pesticides, sulphate or other agricultural-related compounds. Alongside LTEQ a job-exposure matrix (JEM) designed by Brouwers et al. (2009) was applied to assess the occupational exposure to potential known health hazards compounds difficult to access only with self-report questionnaires. Hence, the unlikely, possible or probable risk of exposure to pesticides, organic solvents and metals on the workplace was assessed for each participant. JEM includes 353 job titles linked with the probability of exposure for each group of compounds.

2.4. Biological sample collection

Peripheral blood samples were obtained in the morning, transported in a cooler (4 °C, transport within 30 min maximum) and immediately processed for the different methodologies used in the study. Aliquots of mononuclear cells isolated from BD Vacutainer® CPT™ tubes with sodium citrate (Becton, Dickinson and Company) were cryopreserved in freezing medium (90% of FBS, 10 % DMSO) at a concentration of about 10^6 cells/mL and stored at −80 °C until analysis. All samples were coded and analysed under blinded conditions.

2.5. Comet assay

The alkaline comet assay was performed according to Singh et al. (1988) with minor modifications (Costa et al., 2008), using the medium-throughput comet assay 12-Gel Comet Assay Unit ™ (Severn Biotech Ltd) (Abreu et al., 2017). Briefly, 5 µL of cells suspended on 0.6% (w/v) low-melting point agarose were dropped onto a frosted slide pre-coated with 1% normal melting point agarose, two mini gels were prepared per subject. The gels were allowed to set for 5 min at 4 °C and then immersed in cold lysis solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris-base, 0.25 M NaOH, pH 10; 1% Triton X100) for at least 60 min in the dark at 4 °C. Slides were then immersed in cold alkaline electrophoresis solution (1 mM Na_2EDTA, 300 mM NaOH, pH 13) and left for 30 min in the dark to allow DNA unwinding. After this period, slides were placed on a horizontal electrophoresis tank and covered with 1.2 L cold alkaline electrophoresis solution. Electrophoresis was carried out for 20 min at approximately 1 V/cm. Subsequently, slides were washed with cold PBS for 10 min and rinsed in distilled water for further 10 min. Mini-gels were fixed by immersing slides in 70% ethanol for 15 min and in 96% ethanol for further 15 min. Slides were left to dry horizontally overnight at room temperature in the dark. Dried slides were stained with SYBR®Gold (Invitrogen™) in a bath with agitation at the dilution recommended by the manufacturer (20 u L of SYBR® Gold diluted in 25 mL of TE buffer). After 30 min, SYBR®Gold solution was removed and slides were rinsed twice with water, left to dry at room temperature and stored until scoring. For scoring one drop of water was put onto each mini-gel and the slide covered with coverslip. Microscopic analyses were performed blindly by the same reader on a Nikon Eclipse E400 Epi-fluorescence microscope (G2A filter, Nikon C-SH61). The semi-automated image analysis system Comet Assay IV (Perceptive Instruments, UK) was used for image capture and analysis. A total of 150 cells were scored for each subject. The percentage of DNA in the comet tail (%TDNA) was the DNA damage parameter chosen to describe comet formation.

2.6. Enzyme-modified comet assay

Oxidative DNA damage was assessed through comet assay as described by Azqueta and Collins (2013). Formamidopyrimidine DNA glycosylase (FPG) was the enzyme selected to measure the amount of DNA oxidised purines. FPG (8000 units/mL) was purchased by New England Biolabs® and used according to manufacturers' instructions. In summary, after lysis the slides for the enzymatic treatment were washed three times (5 min each) with buffer F (0.1 M KCl, 0.5 mM Na_2EDTA, 40 mM HEPES, 0.2 mg/mL BSA, pH 8). Gels were then incubated for 30 min at 37 °C with the enzyme or with buffer F alone. The next steps, unwinding and electrophoresis, were performed according to the comet assay classical version described above in Section 2.5. Net FPG-sensitive sites were calculated by subtracting the %TDNA values from buffer and enzyme-incubation.

2.7. γH2AX assay

H2AX phosphorylation was assessed through γH2AX assay according with the protocol previously described by Sanchez-Flores et al. (2015). Shortly, the cell suspensions were thawed, centrifuged and the supernatants were removed. The pellets were suspended in culture medium with 1% phytohaemagglutinin and the cells were incubated for 24 h at 37 °C. Fixation with 1% p-formaldehyde and 70% ethanol followed, and cells were then incubated with anti-human γH2AX-Alexa Fluor 488-conjugated antibody (Becton Dickinson) and stained with propidium iodide (PI). The analysis was performed in a FACSCalibur flow cytometer (Becton Dickinson) with Cell Quest Pro software (Becton Dickinson). A minimum of 10,000 events were acquired in the region of lymphocytes, and data from both FL1 (γH2AX-Alexa Fluor 488) and FL2 (propidium iodide) detectors was obtained. The percentage of gated events positive for both γH2AX and PI was calculated regarding the total lymphocytes gated and indicated as %γH2AX.

2.8. Mercury blood levels (B-Hg) evaluation

Total mercury concentrations were determined in 200 µL of whole blood by atomic absorption spectrometry with thermal decomposition and gold amalgamation in a NIC-MA-3000 equipment (Nippon Instruments Corporation, Japan). Calibration Standards were prepared from ultra-pure mercury reference standard solution (1000 ppm, Spectro ECON; Chem-Lab) and stabilized with a solution of L-Cysteine (> 98%, TCI Chemicals). All samples were analysed in triplicate and the relative standard deviation (%RSD) was always below 10% (mean ± stdev: 3.1 ± 1.78). The certified reference material (CRM) Seronorm® – Trace elements whole blood L-1 (Ref 210105, Lot 1406263) was used for quality control, being analysed every ten samples. The relative standard deviation was below 5%. The obtained results (1.35 ± 0.06 ng.g-1) are within the certified range (1.18–1.77 ng g^{-1}).

2.9. Statistical analysis

A general description of the study population was performed through univariate analysis. The distribution within the study groups of socio-demographic, lifestyle factors and environmental exposure-parameters was evaluated with the Student's *t*-test or one-way analysis of variance (ANOVA) for continuous variables and the Pearson's Chi-

Table 1
Characteristics of the study population.

	Total	Robust	Pre-frail	*p-value*	Frail
Total individuals N (%)	71 (100.0)	32 (45.1)	32 (45.1)		7 (9.8)
Gender N (%)					
Females	49 (69.0)	20 (62.5)	22 (68.8)	> *0.05*[b]	7 (100.0)
Males	22 (31.0)	12 (37.5)	10 (31.3)		–
	p > 0.05[b]				
Age (years-old)[a]	74.9 ± 6.6	72.3 ± 5.7	76.4 ± 6.3	< *0.05*[c]	80.3 ± 7.4
	p < 0.05[c]				
Smoking habits N (%)					
Never smokers	53 (74.6)	22 (68.8)	24 (75.0)	> *0.05*[b]	7 (100.0)
Ever-smokers	18 (25.4)	10 (31.3)	8 (25.0)		
	p > 0.05[b]				
Years smoking[a]	5.8 ± 12.0	7.3 ± 12.3	5.4 ± 12.8	> *0.05*[d]	–
Years stop smoking[a]	30.2 ± 14.1	29.0 ± 10.3	31.6 ± 18.5	> *0.05*[c]	–
Second-hand smokers N (%)	19 (26.8)	5 (15.6)	13 (40.6)	< *0.05*[b]	1 (14.3)
	p = 0.055[b]				
Medication daily intake[a]	4.4 ± 2.9	3.7 ± 2.7	4.6 ± 2.5	> *0.05*[d]	7.0 ± 4.0
	p < 0.05[d]				
BMI[a]	28.1 ± 3.7	27.8 ± 2.7	28.5 ± 4.6	> *0.05*[c]	28.0 ± 3.4
	p > 0.05[c]				
Nutritional status N (%)					
Normal nutritional status	62 (87.3)	32 (100.0)	26 (81.3)	< *0.05*[b]	4 (57.1)
At risk of malnutrition	9 (12.7)	–	6 (18.8)		3 (42.9)
Malnourished	–	–	–		–
	p < 0.05[b]				
Index of basic activities of daily living (ADL) N (%)					
Independence	52 (73.2)	31 (96.9)	20 (62.5)	< *0.05*[b]	1 (14.3)
Mild dependence	15 (21.1)	1 (3.1)	11 (34.4)		3 (42.9)
Moderate dependence	3 (4.2)	–	1 (3.1)		2 (28.6)
Severe dependence	1 (1.4)	–	–		1 (14.3)
Total dependence	–	–	–		–
	p < 0.05[b]				
Scale of instrumental activities of daily living (IADL) N (%)					
Independence	56 (78.9)	31 (96.9)	24 (75.0)	< *0.05*[b]	1 (14.3)
Mild dependence	9 (12.7)	1 (3.1)	7 (21.9)		1 (14.3)
Moderate dependence	2 (2.8)	–	–		2 (28.6)
Severe dependence	3 (4.2)	–	1 (3.1)		2 (28.6)
Total dependence	1 (1.4)	–	–		1 (14.3)
	p < 0.05[b]				

[a] Mean ± standard deviation.
[b] Chi-square test.
[c] t-student test or OneWay ANOVA.
[d] Mann – Whitney *U* test or Kruskal – Wallis.

square test for categorical or nominal variables. The distribution of the variables was compared with the normal distribution by means of the Kolmogorov–Smirnov goodness-of-fit test (confirmed by Shapiro–Wilk test). To achieve a better approximation to the normal distribution a transformation of the data was applied: logarithmic for B-Hg and H2AX phosphorylated (γH2AX) and square root to the oxidative DNA damage parameter (Net-FPG). The effect of frailty status, lifestyle factors and environmental exposure-parameters on the biological endpoints studied was evaluated by one-way ANOVA and Student's *t*-test. Associations between variables were analysed by Spearman's rank correlation. Missing individuals on LTQE (Table 2, Section 3.2) were due to partially answered questionnaires or no participation.

The level of statistical significance was set at 0.05. All analyses were performed using the IBM SPSS software package V. 25 (SPSS, Inc).

3. Results

3.1. Study population

The general characteristics of the study population are described in Table 1. Of the 71 participants, 45.1% were classified as robust, 45.1% as pre-frail (the transitory state of the syndrome) and 9.8% as frail. Since the number of frail individuals was limited, to better understand the influence of the variables in the transition from robust to a frail phenotype the statistical significance between only robust and pre-frail groups is also shown. Age was found to be significantly different between groups, increasing with the transition of the syndrome from robust to frail subjects. Since there were no current smokers in the study population smoking habits were established as never smokers and ever-smokers. Former-smokers had quit smoking for at least 30 years ago. No differences were found between robust and pre-frail groups concerning this endpoint. However, a significant difference was found regarding exposure to second-hand smoke between robust and pre-frail subjects. Second-hand smokers were more prevalent among pre-frail compared to robust subjects. Furthermore, including frail subjects (n = 7) a borderline significance was found, confirming that second-hand exposure to smoke (tobacco) may be an important variable to explore in further studies. In our study population, there were significant differences between groups with an increase on the number of different medication taken per day from robust (3.7 ± 2.7) to pre-frail (4.6 ± 2.5) and to frail (7.0 ± 4.0) subjects. The daily intake ranged between one to twelve different drugs, with all participants reporting intake of medication (data not shown). Statistically significant differences were found concerning nutritional status, ADL and IADL scales. There was a clear increase in the risk of malnutrition and dependence index in the transition from a robust to a frail phenotype. No differences were found between groups on the cognitive status evaluated by the Mini-Mental State Examination (data not shown). No subjects included in the study presented severe cognitive dysfunction confirming the ability and responsiveness of the subjects evaluated.

Table 2
Environmental exposure-parameters in general study population and frailty-phenotype groups.

	Total individuals (n = 55)		Robust (n = 25)		Pre-Frail (n = 24)		*p-value*	Frail (n = 6)	
	Yes	No	Yes	No	Yes	No		Yes	No
Environmental exposure									
Home N (%)									
Use of fireplace	13 (23.6) p > 0.05[a]	42 (76.4)	7 (28.0)	18 (72.0)	6 (25.0)	18 (75.0)	> *0.05*[a]	–	6 (100.0)
Use of pesticides	18 (32.7) p > 0.05[a]	37 (67.3)	9 (36.0)	16 (64.0)	7 (29.2)	17 (70.8)	> *0.05*[a]	2 (33.3)	4 (66.7)
Habits of ventilation	55 (100.0) p > 0.05[a]	–	25 (100.0)	–	24 (100.0)	–	> *0.05*[a]	6 (100.0)	–
Sources of local pollution N (%)									
Farming operation	28 (50.9) p > 0.05[a]	27 (49.1)	15 (60.0)	10 (40.0)	10 (41.7)	14 (58.3)	> *0.05*[a]	3 (50.0)	3 (50.0)
Livestock farming	10 (18.2) p > 0.05[a]	45 (81.8)	6 (24.0)	19 (76.0)	3 (12.5)	21 (87.5)	> *0.05*[a]	1 (16.7)	5 (83.3)
Industrial area/factories	16 (29.1) p > 0.05[a]	39 (70.9)	9 (36.0)	16 (64.0)	5 (20.8)	19 (79.2)	> *0.05*[a]	2 (33.3)	4 (66.7)
Food source N (%)									
Water from well/spring	6 (10.9) p < 0.05[a]	49 (89.1)	6 (24.0)	19 (76.0)	–	24 (100.0)	< *0.05*[a]	–	6 (100.0)
Home-produced vegetables	18 (32.7) p < 0.05[a]	37 (67.3)	14 (56.0)	11 (44.0)	4 (16.7)	20 (83.3)	< *0.05*[a]	–	6 (100.0)
Particular fruit consumption	12 (21.8) p > 0.05[a]	43 (78.2)	8 (32.0)	17 (68.0)	4 (16.7)	20 (83.3)	> *0.05*[a]	–	6 (100.0)
Previous farming activity N (%)									
Pesticides use	5 (9.1) p > 0.05[a]	50 (90.9)	3 (12.0)	22 (88.0)	2 (8.3)	22 (91.7)	> *0.05*[a]	–	6 (100.0)
Sulphate use	9 (16.4) p > 0.05[a]	46 (83.6)	7 (28.0)	18 (72.0)	2 (8.3)	22 (91.7)	> *0.05*[a]	–	6 (100.0)

[a] Chi-square test.

3.2. Lifetime exposure questionnaire (LTEQ)

Results from LTEQ for the study population according to frailty phenotype are described in Table 2. The use of fireplace, pesticides (in plants and/or animals) and insufficient home ventilation are factors than can influence the indoor air quality, therefore a positive answer to these parameters was considered a potential risk of environmental exposure to pollutants (e.g. particulate matter). There were two exposure parameters significantly associated with the frailty phenotypes: the consumption of water from wells or springs and the consumption of home-produced vegetables. A higher number of robust individuals reported to consume more vegetables from this source compared to pre-frail and/or frail individuals. Regarding the use of water from wells or spring water, more robust individuals also reported to consume water from these sources. Household proximity to traffic was also assessed. The level of exposure was categorised according to the intensity of traffic reported by participants as: not intense (low intensity), intense and highly intense. Nevertheless, no significant differences were found in the number of subjects classified as robust, pre-frail or frail living in those areas (data not shown). Considering occupational exposure, this information was matched and linked to a JEM (Brouwers et al., 2009) which allowed to categorise the probability of exposure to "pesticides", "organic solvents" and "metals" as unlikely, possible and likely to occur. No significant differences were found associating the likelihood of exposure to the three contaminants and the frailty phenotypes (data not shown).

3.3. Biological endpoints

Fig. 1 shows the results on different biologic endpoints by frailty phenotypes. According to the univariate analyses, no significant influence of frailty status was obtained on primary DNA damage, oxidative DNA damage, H2AX phosphorylation and B-Hg levels.

Associations between biological endpoints tested were not found according to Spearman's correlation. Nevertheless, the variable "medication daily intake" was the only significant correlation obtained for the association with %γH2AX ($r = 0.267$, $p < 0.05$).

3.4. Biological endpoints vs exposure parameters

Data on the influence of exposure-related factors in the biomarkers studied are presented in Table 3. Since the number of frail individuals was limited, statistical analysis was performed only with robust and pre-frail group. The levels of primary DNA damage were decreased within both groups of individuals who consumed home-produced vegetables. Regarding H2AX phosphorylation an increase in the levels was found for former smokers within both groups, and in home-produced vegetables consumers and pesticide users within the pre-frail group.

As for the B-Hg levels, only a borderline difference was seen for individuals who consumed home-produced vegetables within the robust group.

No significant differences were observed between robust and pre-frail groups when studying the influence of exposure parameters on the biomarkers evaluated.

4. Discussion

As the life expectancy increases globally it is also expected that the prevalence of frailty and its consequences will follow the ageing trend and display higher rates (Ahmed et al., 2007). An early diagnosis of the syndrome and timely interventions are crucial to prevent frailty negative outcomes (e.g.: disability, institutionalisation, death). In an attempt to revert this scenario several studies have been deepen the knowledge on frailty (Rodriguez-Artalejo and Rodriguez-Manas, 2014). Considering frailty complexity, being a combination of several possible causal effects, namely, biological, physiological, social, psychological and environmental changes, the specific risk factors are yet to be determined (Pickard et al., 2019). Hence, in this pilot study, the effect of environmental and occupational exposures during the life course and its

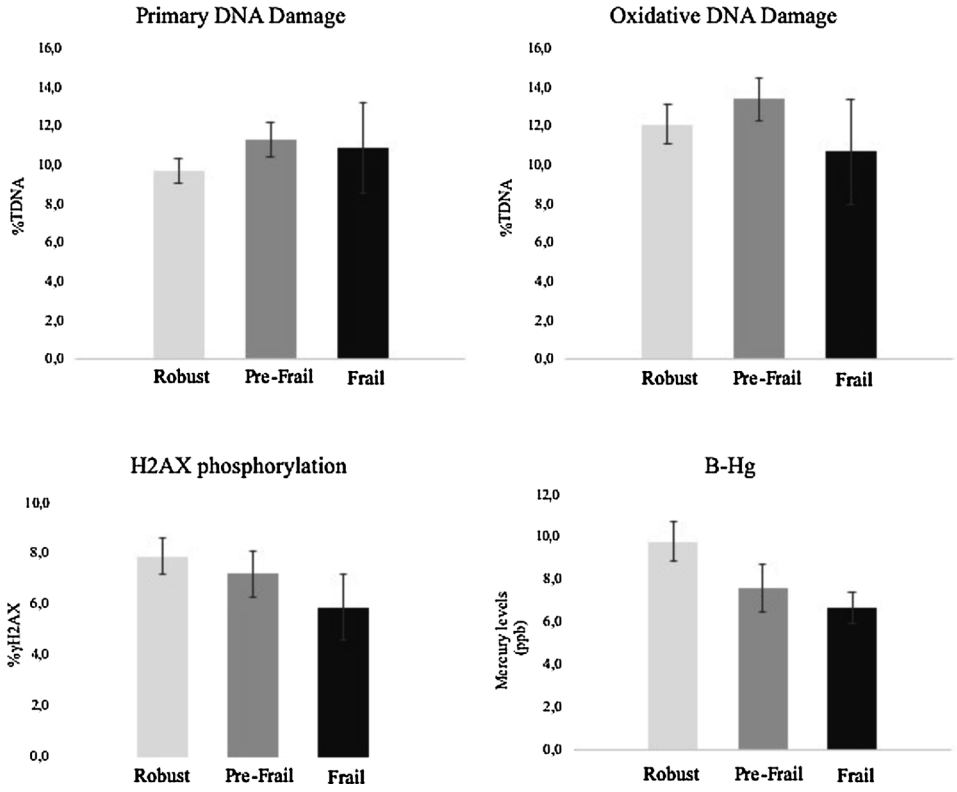

Fig. 1. Results of the biomarkers analysed by the study groups classified according to frailty status (univariate analysis). Bars represent the mean ± standard error.

relationship with frailty prevalence in a population aged 65 years and over was studied. If addressed early, frailty can be prevented or reverted, for that reason, we also searched for some early effect biomarkers, namely primary DNA damage, oxidative DNA damage and H2AX phosphorylation, and an exposure endpoint, the B-Hg levels.

We found an equal percentage of robust and pre-frail subjects and 10% of frail individuals in our study population of older community dwelling adults. This finding is in accordance with a study performed

Table 3
Influence of exposure parameters on the biomarkers evaluated and frail groups.

Biomarker	Exposure parameter	Robust			Pre-frail		
		n	mean ± SE	*p-value*	n	mean ± SE	*p-value*
Primary DNA damage	Smoking			$p > 0.05$[a]			$p > 0.05$[a]
	Never-Smoker	22	9.9 ± 1.2		24	10.8 ± 0.9	
	Ever-Smoker	10	9.3 ± 0.9		8	12.9 ± 2.1	
	Vegetables intake (home-produced)			$p < 0.05$[a]			$p < 0.05$[a]
	Yes	14	8.7 ± 0.6		4	7.7 ± 1.1	
	No	11	11.5 ± 1.2		20	13.1 ± 1.1	
	Pesticides use			$p > 0.05$[a]			$p > 0.05$[a]
	Yes	3	7.9 ± 0.6		2	14.3 ± 6.9	
	No	22	10.2 ± 0.8		22	12.0 ± 1.0	
H2AX phosphorylation	Smoking			$p < 0.05$[a]			$p < 0.05$[a]
	Never-Smoker	22	6.9 ± 0.7		24	5.9 ± 0.6	
	Ever-Smoker	10	9.9 ± 1.2		8	11.2 ± 3.0	
	Vegetables intake (home-produced)			$p > 0.05$[a]			$p < 0.05$[a]
	Yes	14	9.1 ± 1.0		4	13.5 ± 5.8	
	No	11	8.2 ± 1.0		20	6.9 ± 0.7	
	Pesticides Use			$p = 0.053$[a]			$p < 0.05$[a]
	Yes	3	12.8 ± 2.4		2	23.3 ± 7.4	
	No	22	8.1 ± 0.7		22	6.6 ± 0.5	
B-Hg levels	Smoking			$p > 0.05$[a]			$p > 0.05$[a]
	Never-Smoker	22	9.6 ± 1.3		24	7.6 ± 1.4	
	Ever-Smoker	10	10.3 ± 1.1		8	7.6 ± 1.4	
	Vegetables intake (home-produced)			$p = 0.054$[a]			$p > 0.05$[a]
	Yes	14	11.5 ± 1.3		4	6.3 ± 0.6	
	No	11	7.9 ± 2.0		20	8.6 ± 2.4	
	Pesticides Use			$p > 0.05$[a]			$p > 0.05$[a]
	Yes	3	11.1 ± 2.7		2	8.4 ± 2.3	
	No	22	9.8 ± 1.3		22	6.5 ± 0.7	

[a] *t*-Student test.

by Fried and colleagues (Fried et al., 2001) in a cohort of 5317 older adults in which was reported a frailty prevalence of 46.4% robust, 46.6% pre-frail and 6.9% frail consistent with the data observed in our study: a low prevalence of frailty, and a similar prevalence of non-frailty (robust) and pre-frailty. Most of the studies found in the literature regarding frailty prevalence are focused in Northern American population and most recently in the Asian population (Lage et al., 2018; Kojima et al., 2017). Santos-Eggimann et al. conducted a cross-sectional analysis to evaluate frailty in ten European countries and the prevalence of frailty reported was of 42% for pre-frailty and 17% for frailty (Santos-Eggimann et al., 2009). Our data with 71 individuals shown estimates closer to the obtained in other European studies. However, it should be noted that any effort to compare prevalence estimates across studies should be carefully conducted, since the definitions of the frailty criteria, distribution of confounders (e.g., age and sex), and exclusion criteria may differ substantially between studies (Collard et al., 2012; Fernández-Garrido et al., 2014; Santos-Eggimann et al., 2009).

Socioeconomic factors, such as gender and age have been related with the frailty syndrome in several studies (Ahmed et al., 2007). In the present study, higher rates of frailty were observed in older individuals when compared to younger older adult participants. Previous studies reported a correlation between frailty status and older ages (Collard et al., 2012). In fact, age was significantly correlated with frailty status. Important to note is that despite several studies reports age as a possible factor that influences the incidence of frailty syndrome (Morley et al., 2013; Rodriguez-Artalejo and Rodriguez-Manas, 2014), the chronological age *per se* cannot justify the development of frailty syndrome, as observed in several studies by the heterogeneity verified within groups with same age (Dent et al., 2016).

Our data relating the daily intake of medicines with frailty status is consistent with other reports (Abreu et al., 2019; Chen et al., 2019). Polypharmacy is especially common in older adults, the increased consumption of potentially inappropriate medication in this age-group is an emergent concern in Europe and around the world (Fialova et al., 2019). Also, similar to our findings regarding the risk of malnutrition and increased dependence in functional status with frailty can be found in the literature (Abreu et al., 2019; Chen et al., 2019; Lorenzo-Lopez et al., 2017).

Tobacco smoke contains a high number of mutagenic and carcinogenic substances, such as benzene, arsenic and formaldehyde. Epidemiologically it has been associated with a higher risk for human respiratory diseases and cancer development (IARC, 2004). All subjects in the study population were non-smokers but 25.4% reported to be former-smokers. No significant difference was found between the prevalence of ever-smokers and never-smokers between frailty groups. On the other hand, exposure to second-hand smoke shown statistical differences between phenotype-frail groups being more prevalent in the pre-frail group than in the robust group (40.6% vs 15.6%). Our finding is consistent with García-Esquinas et al. (2015b) who performed a study to evaluate the association between second-hand smoke and frailty syndrome (assessed by Fried's Model) in non-smoking older adults ($n = 2059$; ≥ 60 years old). Results of the study showed an increased frequency of frailty in subjects living with two or more smokers at home.

A number of studies have suggested that environmental exposures may be linked with the development of age-related disorders, such as AD (Alzheimer's Disease) (Bakulski et al., 2012; Yegambaram et al., 2015). It was shown that frailty declines the pharmacokinetics (body response to environmental xenobiotics) and pharmacodynamics (action of xenobiotics to the body) functions (Geller and Zenick, 2005). Since frailty is a multifactorial syndrome with no causal effects determined (Dent et al., 2016) environmental exposures should also be considered when investigating risk factors related to this syndrome. Using the LTEQ no particular exposure parameter was significantly associated to a frailty phenotype, with the exception of home-produced vegetables and water from well/spring consumption. We found a higher prevalence of robust individuals among subjects reporting the consumption of vegetables home-produced compared to pre-frail and/or frail individuals. A study of Garcia-Esquinas et al. (2016) referred that the consumption of fruit and vegetables may be a protective factor against frailty, it was verified that consuming three daily portions of fruit and two daily portions of vegetables were strongly associated with lower short-risk of frailty in a dose response manner (Garcia-Esquinas et al. 2016). In this regard, it might be safe to speculate that subjects producing or having access to home-produced vegetables may consume it more often, in a variable and seasonal manner than those who have no easy access. Regarding the association found for water from well/spring consumption no conclusions should be withdrawn given the limited size of the population.

Some previous studies have related the exposure to metals with age-related disorders, namely AD and Parkinson's Disease, showing the role that these environmental pollutants may play in these diseases' aetiology (Bakulski et al., 2012). A study from García-Esquinas et al. (2015a) related the environmental exposure to lead and cadmium with frailty syndrome, by assessing the levels of lead in blood and cadmium in urine. The results of that study revealed a positive dose-response association between frailty and blood lead concentrations, but for cadmium no relation with frailty was found (García-Esquinas et al., 2015a). In the present study we assessed the B-Hg levels, however, no significant association was observed. The past occupational exposure to metals was evaluated via LTQE, but also no correlation was found, probably due to the limited size of the population. To our knowledge few studies were carried out considering environmental and occupational exposures and frailty syndrome. Hence, it will be interesting to explore whether the data from this pilot study replicates in a larger population.

No significant differences were observed for either, primary DNA damage, oxidative DNA damage and H2AX phosphorylation. In a larger Spanish population similar results were found for comet assay endpoints (Marcos-Perez et al., 2019; Valdiglesias et al., 2019) as for H2AX phosphorylation, increased levels of H2AX phosphorylation with increasing frailty status were observed (Valdiglesias et al., 2019). A recent review compared the epidemiological studies that linked frailty with alterations at cellular level, namely oxidative stress, genomic instability and DNA damage and repair biomarkers (Sanchez-Flores et al., 2017). No link with biomarkers of genomic instability were found, but variations in oxidative stress, such as vit- E (Ble et al., 2006), glutathione and oxidized/reduced glutathione ratio (Serviddio et al., 2009) and biomarkers of lipid peroxidation (Collerton et al., 2012) were often associated to frailty status. If oxidative stress arises as a consequence of frailty or as product of a bidirectional relation where the presence of one of them increases the risk of the other is still unknown (Sanchez-Flores et al., 2017).

The relation between some of the data collected in LTEQ on the levels of genotoxicity endpoints and in the B-Hg levels was analysed (Table 3). Significant differences were found regarding some exposure-related parameters, namely, smoking habits, the intake of home-produced vegetables and the use of pesticides (non-occupational). Within the robust and pre-frail groups the levels of primary DNA damage were decreased in both groups of individuals who consumed home-produced vegetables. As mentioned herein, this effect may be related with the protective factor against frailty of vegetables as reported in the Garcia-Esquinas et al. (2016) study, probably these individuals consumed more vegetables than those that had to purchase them. As for the B-Hg levels, only a borderline non-significant increase was seen for individuals who consumed home-produced vegetables within the robust group. Regarding H2AX phosphorylation an increase in the levels was found for former smokers within both groups. Other relevant differences observed were the increased levels of H2AX phosphorylation in home-produced vegetables consumers and pesticide users within the pre-frail group. The γH2AX assay evaluates double DNA strand breaks, a specific kind of DNA lesion, that if persistent in time might be unrepairable

(Sedelnikova et al. 2008). This molecular event can have deleterious consequences for the genome and for the cell causing loss of genetic information. For that reason, the accumulation of DNA double strand breaks can lead to cellular senescence and organismal aging. In fact, Silva et al. (2014) reported an in nuclear expression levels of γH2AX in individuals with AD, an age-related disorder, when compared to healthy individuals. The obtained data suggests possible unrepairable damage, possibly due to pesticides exposure. Moreover, a study using human cell lines, representing potential target tissues of food contaminants, confirm the DNA damage induced by pesticides to these cells using the γH2AX assay (Graillot et al., 2012). Considering the B-Hg levels, the borderline increase in individuals that consume home-produced vegetables can be related with the possible bioaccumulation of this metal in vegetables probably by contamination of the soil or water. However, regarding this data we always should be cautious and have in consideration the limited number of individuals enrolled in this pilot study.

5. Conclusions

Data from this pilot study shows the importance of including the exposure-related factors when estimating frailty prevalence or investigating potentially factors promoting this syndrome onset. Nevertheless, we should take into account the reduced population size, and that further studies are necessary to corroborate whether the current findings are consistent and reproducible in larger sample sizes. Always having in mind that the goal is improving the healthcare and quality of life of older adults by implementing preventive actions and specialising care, thus reducing healthcare costs.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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