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Life-course exposure and frailty syndrome in older adults

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LIFE-COURSE EXPOSURE AND FRAILTY SYNDROME IN OLDER ADULTS

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SCIENTIFIC PRODUCTION

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[1] Diagnosis, Monitoring and Prevention of Exposure-related Non-communicable Diseases in living and working environments

ABSTRACT

One area of health concern is reducing the burden of environmentally induced disease in populations that may be more susceptible to the effects of exposures to contaminants. The potential to reduce the prevalence of some major diseases is driving research to understand the totality of exposures over the course of our lifetimes. Older adults are well-recognized susceptible subpopulation. Health status in older adults is complex and multidimensional. One metric is frailty, a state of increased vulnerability to stressors, characterized by decreased physical and mental functioning and an increased risk for poor health outcomes. The European Commission has appointed Ageing one of the main priorities in the next Horizon 2020 Framework Program, the prevention of frailty in old age is one of the key actions identified.

The aim of the present study is to build and apply a life-course exposure questionnaire and study the association with DNA basal damage and oxidative damage endpoints with frailty syndrome, contributing to the knowledge of the mechanistic pathways and syndrome aetiology.

A total of 61 voluntary individuals aged 65 and over were involved in the study from senior recreational community associations and day care centres, located in metropolitan region of Oporto. Frailty assessment was performed using Fried's frailty model and the individuals were classified as robust, pre-frail or frail. Life-time exposure was evaluated by a self-reported life-course exposure questionnaire and a job exposure matrix application. DNA basal damage and DNA oxidative damage endpoints were measured through comet assay in whole blood.

Study population was classified as 47.5% robust, 49.2% pre-frail and 3.3% frail. A relation between the prevalence frailty with age and with gender was observed, with women and older elderly displaying higher rates of frailty. A relation between frailty status and second-hand smokers was found, since higher prevalence of exposure to tobacco smoke was found in pre-frail group (23.3%) when compared with the robust individuals (10.4%). Associations between frailty status and consumption of home-produced vegetables were found, with robust individuals consuming more home-produced vegetables (71.4%) from this source compared to pre-frail individuals (28.6%) that eat those aliments. Furthermore, associations between the consume of these vegetables and DNA damage in robust groups were found, since the robust individuals that include these aliments in their diet showing lower DNA damage than robust individuals that not consumption those aliments from particular produced sources. Regarding the effects of the variables studied, a significant influence

was found on the genotoxic endpoints for gender and age within the robust group ($p<0.05$). Thus, significant differences were observed between the basal damage between robust females and males and between the oxidative damage between earlier age group and 75-84 age group. Lastly, also a relation was verified between the role of current exposures and the DNA damage, regarding household-proximity to farming operation within the robust group. Thus, robust individuals that reported to live near of this activity have higher basal and oxidative damage than those robust individuals that do not live near farming operations ($p<0.05$).

Data obtained provides preliminary information on relations between exposure, frailty syndrome and DNA damage. Further studies need to be performed in order to deepen the knowledge about frailty aetiology and the possible role of life-course exposures, helping to understand how the past may affect the future.

RESUMO

Uma das prioridades no âmbito da saúde é reduzir a carga de doenças em populações que possam ser mais suscetíveis ao efeito das exposições a contaminantes. De modo a reduzir a prevalência de algumas das principais doenças, os investigadores têm trabalhado no sentido de tentar compreender a influência que exposições ao longo do curso de vida possam ter nestas mesmas doenças. A população idosa é considerada uma subpopulação suscetível. O estado de saúde em idosos é complexo e multidimensional. A fragilidade é um estado de vulnerabilidade acrescida, caracterizada pela diminuição da função física e mental e que pode conduzir a hospitalização, a internamento e a incapacidade a longo prazo. A Comissão Europeia definiu o Envelhecimento como uma prioridade do Programa Horizonte 2020, sendo que a fragilidade em idosos é uma das principais ações identificadas.

O presente estudo tem como principais objetivos a construção e aplicação de um questionário de exposição ao longo do curso de vida e a sua relação com o dano no ADN basal e oxidativo, contribuindo para o aprofundamento de conhecimento sobre os mecanismos e etiologia da síndrome.

Um total de 61 indivíduos com idade igual ou superior a 65 anos, participou de forma voluntaria no estudo. Os participantes envolvidos foram recrutados em várias instituições localizadas na região metropolitana do Porto. A fragilidade foi avaliada segundo o modelo fenotípico de Fried, e os indivíduos foram classificados como robustos, pré-frágeis e frágeis. As exposições foram avaliadas através de um questionário de exposição ao longo do curso de vida e da aplicação de uma matriz de exposição ocupacional. O dano basal e oxidativo no ADN foi medido através do ensaio cometa.

Após as avaliações, 47,5% da população estudada foi classificada como robusta, 49,2% como pré-frágil e 3,3% como frágil. Fatores como o género e a idade foram associados com uma maior prevalência de fragilidade, uma vez que tanto as mulheres como os idosos de idade mais avançada apresentaram um estado de fragilidade superior (pré-frágil). Uma relação entre a síndrome de fragilidade e a exposição ao tabaco em fumadores passivos foi observada, com maior prevalência de exposição no grupo pré-frágil (23,3%) relativamente ao grupo robusto (10,4%). Associações entre a síndrome de fragilidade e o consumo de vegetais de produções particulares foram observadas no estudo, uma vez que se verificou que haviam mais indivíduos robustos a consumir vegetais dessa fonte (71,4%), quando comparados com o número de indivíduos pré-frágeis (28,6%) que afirmaram consumir estes mesmos alimentos. Por outro lado, foram encontradas associações entre

o consumo de vegetais de produções locais e o dano no ADN em grupos robustos, uma vez que os indivíduos que incluíam esses alimentos na dieta apresentaram menor dano no ADN do que que os indivíduos do mesmo grupo que não consumiam esses alimentos. Em relação ao efeito das variáveis nos fatores em estudo, tanto a idade como o género influenciaram de forma significativa o dano no ADN dentro do grupo robusto (a idade com influência ao nível do dano basal e o género no dano oxidativo do ADN) ($p<0.05$). Quando à relação entre a fragilidade, a exposição e o dano no ADN, foram observadas diferenças dentro do grupo robusto. Diferenças estatisticamente significativas foram observadas entre a presença de explorações agrícolas perto da zona de habitação e o dano basal no ADN, sendo que os indivíduos que reportaram viver perto desta atividade apresentaram menor dano basal no ADN do que aqueles que afirmaram não morar perto desta atividade ($p<0.05$).

Os dados obtidos fornecem informação sobre as relações entre a exposição, a síndrome de fragilidade e o dano no ADN. Estudos futuros devem elucidar a relação entre a etiologia da síndrome de fragilidade e possíveis exposições ao longo do curso de vida, o que será relevante para perceber de que modo o passado pode afetar o futuro, ajudando na implementação de ações preventivas.

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ABBREVIATIONS AND ACRONYMS

AD	Alzheimer's Disease
ADL	Activities of Daily Living
BMI	Body Mass Index
CGA	Comprehensive Geriatric Assessment
CHS	Cardiovascular Health Study
DMSO	Dimethyl Sulfoxide
EDTA	Ethylenediamine Tetra-Acetic Acid
EPA	Environmental Protection Agency
FPG	Formamidopyrimidine DNA Glycosylase
IADL	Instrumental Activities of Daily Living
LTEQ	Lifetime Exposure Questionnaire
MLTPAQ	Minnesota Leisure Time Physical Activity Questionnaire
OECD	Organisation for Economic Co-operation and Development
PD	Parkinson's Disease
PM	Particulate Matter
PPE	Personal Protective Equipment
SHARE	Survey of Health, Ageing and Retirement in Europe
UDG	Uracil-DNA Glycosylase
VREM	Versión Reducida en Español del Cuestionario de Actividad Física en el Tiempo Libre de Minnesota (Spanish Version of the Minnesota Leisure Time Physical Activity Questionnaire)

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INTRODUCTION

1. Ageing and Frailty Syndrome

The world's population is ageing fast. Population with 60 years old or older is growing at a rate of about 3% per year (United Nations, 2017). Over the next years, a further increase is almost inevitable given the size of the cohorts born in recent decades. This unprecedented phenomenon is due to the increase in life expectancy along with a decrease in fertility index (Lunenfeld and Stratton, 2013).

In the last decades, the increment of lifetime expectancy especially in Europe results of both advancement in social-sanitary conditions and evolution of medicine and technology (Villacampa-Fernandez *et al.*, 2017). On the other hand, the decline in fertility is a global tendency, mostly seen in developed countries as a consequence of the modern way of life. Aspects that may contribute are later-in-life pregnancies and fewer children born per couple. Between the factors that may lead to late pregnancy stands out the increased participation of women in labour market, the improvements in information about methods of conception and contraception and consequently the generalization of efficient contraceptive methods (Valente Rosa and Chitas, 2010). To guarantee the level required for replacement of the population in the long run, known as replacement-level fertility, a fecundity rate of 2.1 per women is necessary (United Nations, 2017). Currently, Portugal has a fecundity rate of 1.2 live births per women, almost two times lower the 2.1 shift value, one of the lowest in Europe, which will result in a 12% decline of the total Portuguese population by 2050 (United Nations, 2017).

At present, 13% of the total world population is 60 years old or over; this percentage in Europe corresponds already to 25%, a quarter of the population (United Nations, 2015). Europe is the "oldest" of the five continents and by 2050 is predicted that this age group will reach 35% of the total European population (United Nations, 2017). This demographic shift offers enormous opportunities as well as great challenges for all societies having clear socio-economic implications such as increases in the cost of healthcare and social support, the number of workers per retiree and development of dependency programs/structures for the elderly. Nowadays, ageing, and its concerns, became a point of growing importance (Rodriguez-Artalejo and Rodriguez-Manas, 2014), the challenge society's face is to ensure that the increase in longevity is accompanied by maintenance of health and quality of life of the elderly population with a parallel reduction of health and social costs. The European Commission is taking proactive measures to tackle the future challenges posed by an ageing population by prioritizing initiatives within Horizon 2020 Program (Rodriguez-Artalejo and Rodriguez-Manas, 2014).

According to recent European statistics by 2030 nearly a quarter of the Portuguese population will be aged 60 or over (Eurostat, 2017) occupying the third place among the countries with the oldest populations in the world (United Nations, 2015). Furthermore, according to the same source, it is expected that by 2050, this population age-group will represent more than 40% of the Portuguese population. At present, Portugal's ageing index is already amongst the top five in Europe with 148,7 of persons 65 years old or over per 100 young persons (under age 15) (Pordata, 2015a, 2016).

In 15 years, Portuguese life expectancy has increased from 76.4 years to 80.6 years at birth (Pordata, 2015b). Life time expectancy differs in a gender dependent way, with women having more lifetime years than men (e.g. 2015, 83.3 vs 77.6 years at birth) (Pordata, 2015b), following world trend. Furthermore, data from the United Nation Prospects (2015) shows that between 2025 and 2030 Portugal will be the second country with lowest total fertility (average number of births) together with Moldavia with around 1.31 (live) births per women, the first will be Singapore with 1.30. The distribution of the Portuguese population by age groups in the years 2000 and 2015 and for 2030 and 2050 is represented in Figure 1. Through these years a progressive change in the population pyramid can be observed with an increase in population in the age groups aged 60 or over and a decrease in younger groups. Research studies on ageing are of paramount importance in Public Health, since an extending lifespan is not (always) synonymous of a healthy life or healthy ageing or quality life (Fries *et al.*, 2011; Rodriguez-Artalejo and Rodriguez-Manas, 2014). In fact, although the European median life expectancy at birth for both sexes is 76.8 years, the estimated median of healthy life expectancy is 68 years (Villacampa-Fernandez *et al*., 2017).

Frailty is an age-related syndrome characterised by a reduction in the physiological reserve required to respond to endogenous and exogenous stressors (Fielding, 2015). More importantly, it has been identified to be the most common condition leading to disability, institutionalisation and death in older adults (Ruiz *et al.*, 2012). Due to the increased vulnerability resulting from frailty, even small disturbances (e.g. variations in ambient temperature) can result in several complications (Clegg and Young, 2011; Ruiz *et al*., 2012). Furthermore, frailty can affect various organ systems and multiple deficits can occur in combination: functional decline (Villacampa-Fernandez *et al*., 2017), sarcopenia (Puts *et al.*, 2005a), neuroendocrine dysregulation (Clegg and Young, 2011) and immune impairments (Puts *et al*., 2005b).

The prevention of frailty syndrome has become an emerging point in European Union (Healthy) Ageing policy, integrating Horizon 2020 as one of the key actions (Rodriguez-

Artalejo and Rodriguez-Manas, 2014). Although the study of frailty may shed light on ageing process, namely why some older adults develop certain disabilities later in life, there are many different concepts for this syndrome and yet no clear definition. Since frailty is a multi-factorial syndrome that comprises among others health, mental health and nutrition, which in turn is linked to environmental, cultural, economic and ethnic considerations different concepts have emerged in the scientific community (Dent *et al.*, 2016). Even though frailty was already perceived among clinical and scientific communities for a long time, it was only in the early 2000's that some authors attempted to describe it, but as a synonym of disability, comorbidity or advanced old age (Fried *et al.*, 2001).

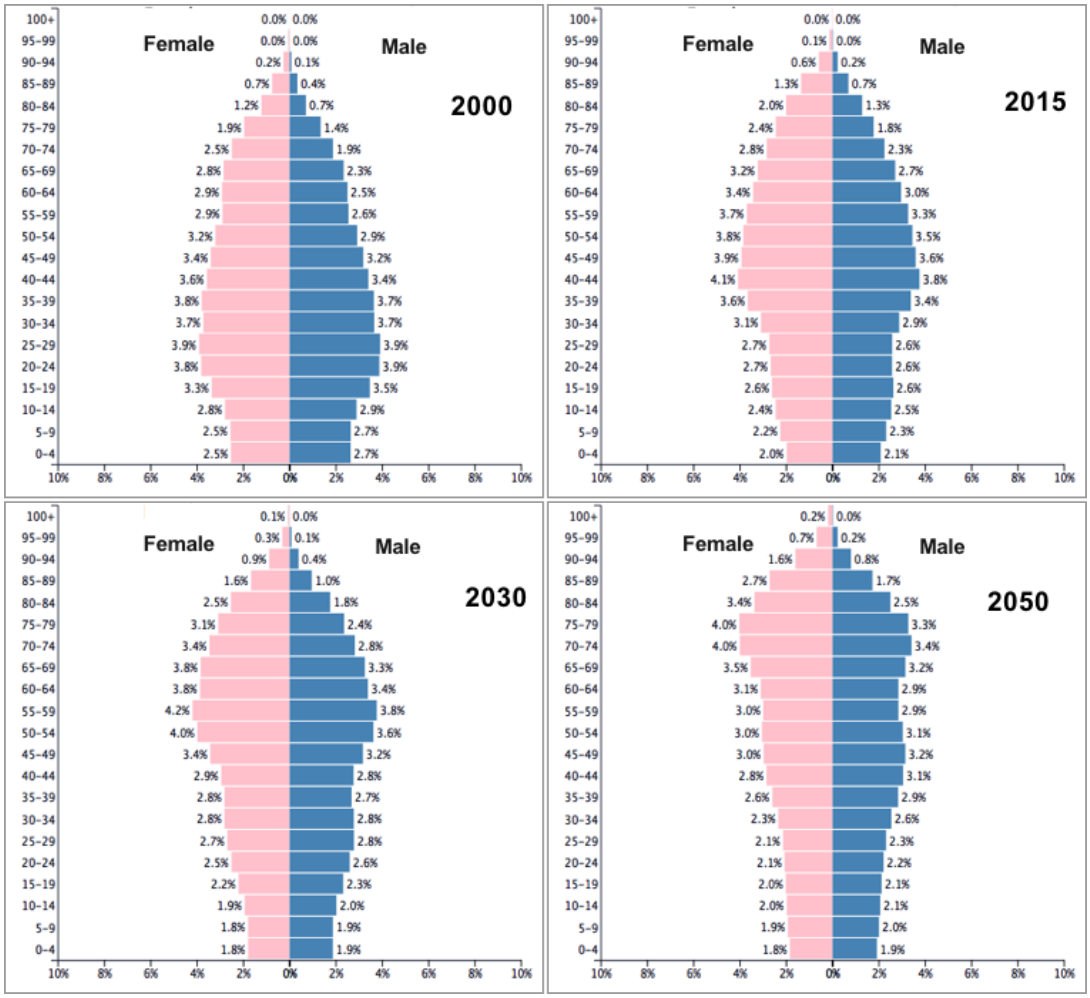

Figure 1. Distribution of population in Portugal, in 2000 and 2015. Prospective distribution of population in Portugal, for 2030 and 2050. Adapted from *www.populationpyramid.net/portugal*.

According to Ahmed *et al.* (2007), disability is the inability to perform activities of daily living (ADL), instrumental activities of daily living (IADL) or difficulty with mobility, so it has no effect on the organ systems contrary to what happens in frail subjects. Furthermore, only 28% of elderly with disability are frail (Ahmed *et al.*, 2007). In 2012, the EU-funded project

'Frailty operative definition - consensus conference (FOD-CC)' established to characterise this emerging syndrome, distinguished frailty from multimorbidity (presence of two or more chronic diseases in an individual). Multimorbidity is perceived individually and consequently the treatment is adapted for each condition while frailty is seen as a whole, which goal is having a successful treatment for the syndrome (Rodriguez-Manas *et al*., 2013). Although both health disorders are common among the elderly, multimorbidity is more prevalent (Morley *et al*., 2013).

Researchers strive to understand what makes the elderly frail, a fact of life that bears strongly on productivity, economic output and health care (Sanchez-Flores *et al*., 2017). The increasing interest in frailty research during the last years (Duarte and Paúl, 2014) is represented in Figure 2, showing the number of annual publications in MedLine/PubMed database (National Library of Medicine, National Institutes of Health, Bethesda, MD, USA) between 2000 and 2016 using as key heading “frailty” and “elderly” using AND operator. The study of frailty sheds light on why many older persons develop disabilities later in life, even if researchers cannot agree on the definition of the concept.

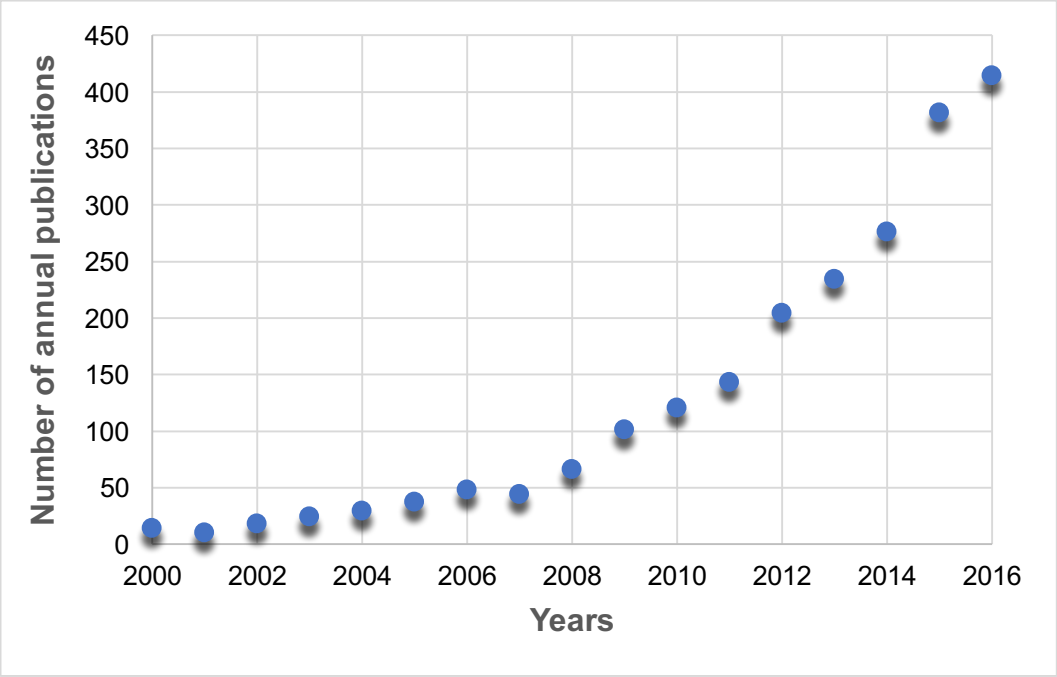

Figure 2. Summary diagram representing the number of publications per year between 2000-2016 in MedLine/PubMed database with subject heading “frailty” and “elderly”.

Nowadays, although there is no gold-standard definition of frailty, there are some agreements among the scientific community, namely: a) the recognition of frailty as a clinical syndrome; b) the differentiation of frailty from disability; c) the possibility of reversion or regression of frailty, and lastly, d) the characterisation of frailty by an increased vulnerability

(Morley *et al*., 2013). Frailty can be also understood as a physical or psychological state or also a combination of both (Morley *et al.*, 2013).

1.1. Frailty Models

Frailty is widely recognized and accepted as a medical syndrome, yet debate remains about the best way to measure it in clinical and research settings. As stated herein, different models of frailty based in different features and scores has emerged, but some have become more popular than others (Ruiz *et al*., 2012; Morley *et al*., 2013). The most popular indices used by clinicians and researchers are the Frailty Index of Rockwood and the Fried's Frailty Phenotype (Collerton *et al*., 2012; Panza *et al*., 2015).

Rockwood and colleagues suggested a multidimensional model to identify frailty where the presence and accumulation of health-related deficits and disorder results in frailty state as an ultimate outcome (Rockwood *et al*., 1999; Mitnitski *et al*., 2001). In this context, the Rockwood Index Model includes social and psychological features such as mood, cognition or incontinence (Collard *et al*., 2012). The index is expressed as a ratio, the number of health deficits existent are divided by the number of health deficits evaluated. For example, in a study with 50 possible health deficits if an individual scores five of these health deficits its frailty index will be 0.1 (Rockwood *et al*., 2005). Rockwood index is well validated and has been applied in several studies including the Survey of Health, Ageing and Retirement in Europe (SHARE). Despite its positive attributes the index is not popular among clinicians since it is time consuming to calculate and apply (Dent *et al*., 2016). Jones *et al*. (2004) proposed a second index derived from the Rockwood Index Model but adding data from a Comprehensive Geriatric Assessment (CGA). A CGA is a global standard clinical assessment for elderly populations that includes medical, nutritional, functional and psychological assessments (Dent *et al.,* 2016). This second index (known as FI-CGA) is a ten-domain index that had originally 14 CGA components but later was extended by Rockwood *et al.* (2010) to include 52 CGA components; deficits in each domain were scored as 0 – no problem, 1 – minor problem or 2 – major problem. Then, frailty was categorized in function of FI-CGA sum: mild (0-7), moderate (7-13) and severe (>13). This index has been used in clinical settings for assessing frailty syndrome and is capable to predict patient response in different areas, such as oncology, immunology or urology (Dent *et al*., 2016).

In 2001, Linda P. Fried and colleagues using data from the Cardiovascular Health Study (CHS) established one of the most widely used and accepted models to assess frailty in

older adults, recognizing frailty as a clinical phenotype (Ensrud *et al*., 2008; Fried *et al*., 2001). In this phenotype-based model, the frailty syndrome is understood and classified according to five criteria: weakness, slowness, low physical activity, exhaustion and weight loss (Fried *et al.*, 2001). Thus, the frailty status in an individual depends on the presence or absence of physical criteria. If an individual manifests three or more components is classified as frail, with one or two is pre-frail and in the absence of criterion is robust (Morley *et al.*, 2013; Duarte and Paúl, 2014). Several studies showed that pre-frailty is a predictive stage of frailty, since pre-frail individuals are more than twice as likely to become frail than robust individuals (Fried *et al*., 2001; Fernandez-Garrido *et al*., 2014).

According to Fried *et al*. (2001) the five criteria - weakness, slowness, low activity, exhaustion and weight loss – are measured as following:

- Weakness is assessed by hand grip strength using a hand-dynamometer, with cut-offs stratified by gender and body mass index (BMI) quartiles;

- Slowness is determined by gait speed, individuals are asked to walk 4.57 meters, the time spent is registered in seconds and stratified by gender and height;

- Exhaustion is evaluated based in the response to two questions from the Center for Epidemiological Studies Depression Scale (CES-D questionnaire) of National Institute of Mental Health (EUA);

- Weight loss, is punctuated by the answer “yes” to the question “Have you lost more than 10 pounds (4.5 kg) in last year unintentionally?” or by the identification of loss of ≥5% body weight in the previous year (follow-up) after a weight examination;

- Low physical activity measurement is based in a self-report for each individual about their own physical activity and exercise, calculating the average kilocalories spent per week, by gender.

Although the loss of weight is one of five criteria assessed, both extremes of BMI seem to be involved in frailty status, with studies associating obesity with frailty development (Ahmed *et al*., 2007; Fernandez-Garrido *et al*., 2014). As previously described, frailty syndrome is a dynamic process that includes multiple components. Fried *et al*. (2001) proposed a “*frailty cycle*” where components of frailty interact between them and undergo different steps of frailty (Figure 3) (Clegg and Young, 2011). Sarcopenia defined as the progressive loss of skeletal muscle mass and strength is pointed as the key element for the advance of frailty

in the elderly (Serviddio *et al*., 2009; Wilson *et al.,* 2017). This event is related with physical function impairment, loss of autonomy and death (Fielding, 2015). The interaction between fibre loss, muscle fibre atrophy and other multiple factors such as nutrition, hormonal system, metabolism and immune system was hypothesised to explain the development of sarcopenia (Clegg and Young, 2011).

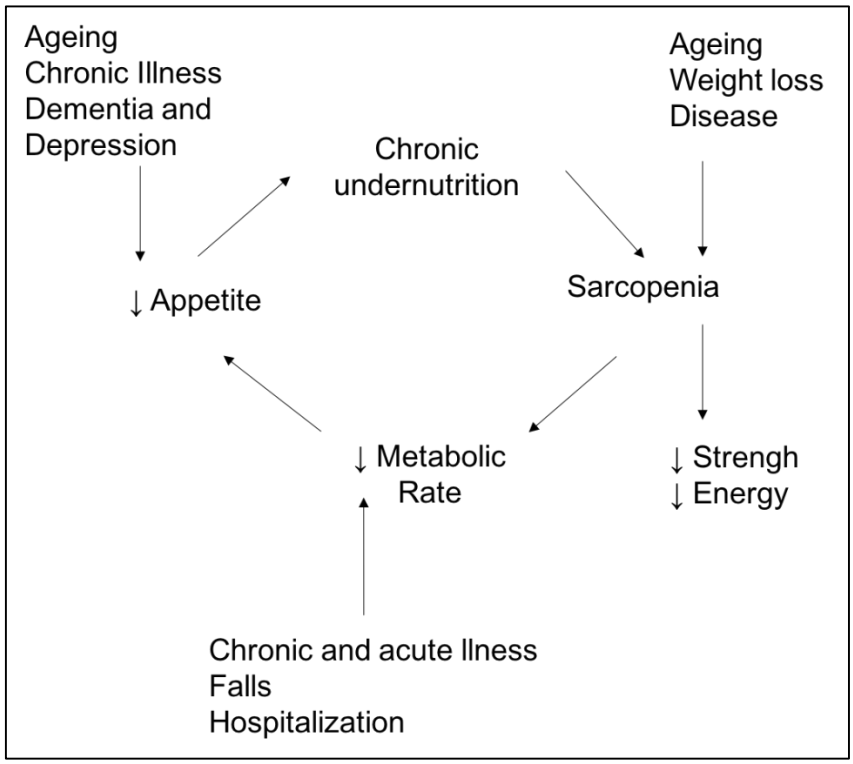

Figure 3: The Frailty Cycle. Illustrative scheme adapted from Ahmed *et al*. (2007) and Clegg and Young (2011).

Fried phenotype model has been proposed for different clinical, research and health care applications namely a) screening elderly populations for frailty syndrome and risk of developing it (Fried *et al*., 2001); b) to distinguish target groups for including randomized tests with the aim of prevent or delaying functional decline and disability (Ferrucci *et al*., 2004); and c) to integrate the comprehensive care for older women (Ensrud *et al*., 2008).

Despite Rockwood's Frailty Index measures more variables than Fried's Frailty model its practical application is difficult in clinical settings due to the extensive number of variables and consequently the phenotypical model became the most popular (Rodriguez-Artalejo and Rodriguez-Manas, 2014; Villacampa-Fernandez *et al*., 2017). Nevertheless, in spite of some conflicting studies, there are data indicating that both models may conduct to similar results. A study of Young *et al*. (2016) performed with twins with the aim of understanding the genetic variance of frailty using the Rockwood Model concluded that the results were

similar to previous studies using Frailty Phenotype. Similarities and differences between the two models are not fully understood (Blodgett *et al*., 2015), and although both can be used to assess the frailty status in older adults the Fried's Phenotype Model seems to be preferred (Zhu *et al*., 2016). The most common disadvantage pointed to Fried's Model is the absence of cognitive and psychological components (Villacampa-Fernandez *et al*., 2017). Another factor that can inhibit its application, especially in clinical settings (e.g. measurement of grip strength) is the use of specific instruments like the dynamometer (Dent *et al*., 2016). Notwithstanding, it remains the most widely used model due to its qualitative and categorical score and its intuitive assessment and simple interpretation by clinicians, researchers and health care professionals (Rodriguez-Artalejo and Rodriguez-Manas, 2014; Dent *et al*., 2016).

1.2. Frailty and Associated Factors

Several authors have studied the possible factors associated with the frailty syndrome (Fried *et al*., 2001). Socio-demographic markers such as gender, ethnic group, education level, economic status, and health status namely, chronic illness and disability have been found to relate to the prevalence of this syndrome in older adults (Ahmed *et al*., 2007).

Various studies report that women display higher rates of frailty when compared with men (Collard *et al*., 2012; Ruiz *et al*., 2012), different hypotheses were given to justify this gender difference. On one hand, taking into account the relation between sarcopenia and frailty, it is possible that the lower average amounts of lean body mass and muscle strength in women can justify this discrepancy among both sexes (Collard *et al*., 2012). Fried *et al*., (2001) stated that sarcopenia may influence the frailty status in women since they are more likely to have a wrong nutritional intake compared with men. On the other hand, Puts *et al*., (2005b) reported a higher frequency of men dying unexpectedly during a follow-up study period when compared with women, while women displayed a progressive health decline. Thus, another possible hypothesis to explain this gender-difference is the life expectancy. Globally, between 2010 and 2015 life expectancy was 69 years for men and 73 years for women (United Nations, 2017). These data show that men statistically have a shorter life expectancy than women and consequently the number of older women in the studied age groups is higher than the number of older men in the same group. Assuming that frailty increases with age, this could explain the difference of rates between genders found in several studies (Collard *et al*., 2012).

As stated herein other factors related with the prevalence of frailty syndrome in older adults include ethnicity, economic status, cognition, nutrition and the presence of comorbidities and/or disability. Some authors reported that Mexicans or southern Europeans with lower socioeconomic status, cognitive dysfunction, poor nutrition and presenting comorbidities or disability are more susceptible to frailty (Ruiz *et al*., 2012). Moreover, these associations were maintained when tested independently. Similarly, Fried *et al*. (2001) observed in their study that frail subjects often shared some features namely being African American, having lower education, lower income, more health problems, such as, higher rate of arthritis, diabetes and pulmonary and cardiovascular diseases, higher rate of comorbid chronic diseases and disability.

Frailty incidence is also related with ageing, with a number of studies showing a correlation between frailty status and older ages. Some authors found high rates of frailty in elderly community-dwellers, as Walston *et al*. (2006) that recorded a frailty rate between 20% to 30% among elderlies over 75 years old. However, there are no conclusive data since the rates may vary in function of the criteria used to classify the frailty status (Collard *et al*., 2012). Nevertheless, it is known that the likelihood to develop frailty increases exponentially in oldest elderly above 80 years (Morley *et al*., 2013). A recent study estimated that frailty rounds 10% in elderly over 60 years and increases for around 25% in people with over 85 years (Rodriguez-Artalejo and Rodriguez-Manas, 2014). Nonetheless, it should be noted that although age may be a factor that influence the incidence of frailty syndrome, frailty occurs independently from chronological age (Dent *et al*., 2016).

Since there are different models of frailty that measure different criteria, it is possible that some results and conclusions obtained in studies using different frailty models are not consistent between them. Thus, prevalence of frailty is not only dependent of features as those previous described but also of the frailty models applied (Collard *et al*., 2012).

A number of different factors that may lead to the condition (or related to frailty incidence) have been suggested, however, a definitive correlation is still missing, as well as, evidence on why it develops in some individuals and not in others (Garcia-Esquinas *et al.,* 2015b; Walston *et al*., 2006). Certain environments, medications, age-related changes, and diseases were some of the factors speculated to make a particular genotype of people vulnerable to frailty (Ahmed *et al*., 2007), well-design studies with larger populations are needed to produce information on this subject.

1.2.1. Frailty and Environmental Health

One area of public health concern is reducing the burden of environmentally induced disease in populations that may be more susceptible to the effects of exposure to contaminants. Older adults are a well-recognized susceptible subpopulation (Risher *et al*., 2010). However advanced age alone may not determine susceptibility. 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. Recent evidence advocates that healthy ageing may be possible, with morbidity compressed to the later years of life (Cohen and Gerber, 2017). Hence, susceptibility associated with advanced 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).

Although all of the previously described factors may influence the prevalence of frailty, is also feasible that environmental exposure plays an important role in this syndrome (Fougere *et al.,* 2015), taking in account that individuals and communities are affected both by genetics and environmental factors (Risher *et al*., 2010). The Environmental Protection Agency (EPA) identified some groups of chemicals that present a risk for elderly, such as metals, pesticides, water contaminants and air particulate matter (PM) (Garcia-Esquinas *et al.,* 2015a). Risk is increased as some of these compounds have the ability to bioaccumulate in target organs during lifetime (Garcia-Esquinas *et al.*, 2015a). Absorption and distribution of some chemicals in human body could depend on the age since elderly suffered major changes in body composition and consequently showed increased impairment to maintain physiological homeostasis (Risher *et al*., 2010; Garcia-Esquinas *et al*., 2015a).

Only few studies have been conducted to explore the association between environmental exposure and frailty syndrome (Garcia-Esquinas *et al*., 2015a,b) and most of the available studies are focused on air pollution (Eckel *et al.,* 2012, Myers *et al.*, 2013). Moreover, studies available in this area were carried out recently, only in the last 5 years. In a study performed by Garcia-Esquinas *et al*. (2015b) was observed an evidence of an association between frailty and second-hand smoke exposure. In other study, a higher risk of frailty development and exposure to $PM_{2.5}$ in hospitalized patients with myocardial infarction was observed, although the study was carried out with a population with 65 years and younger (Myers *et al*., 2013). In addition, it was found that exposure to phthalate may influence grip-hand strength (Kim *et al*., 2016), which may be suggestive of an increase of weakness.

Similarly, a study performed in subjects aged 60 or over indicated a direct dose-response between frailty and the level of lead (Pb) in blood (Garcia-Esquinas *et al*., 2015a). The results showed that when Pb concentration in blood increased 1%, 0.72% of muscle mass was lost (Garcia-Esquinas *et al*., 2015a).

Some age-related disorders, such as Alzheimer's disease (AD) and Parkinson's disease (PD) with unknown aetiology, had been related with environmental exposure.

Research suggests that about 70% of the causes of Alzheimer's disease are genetic and 30% are due to other factors including environmental exposures, smoke, obesity and sedentary lifestyle (Bakulski *et al*., 2012; Yegambaram *et al*., 2015). Most compounds present in the environment have not been tested for their effects in the nervous system, however it is established that the development of AD and PD in elderly could be a consequence of neurologic impairment due to environmental chemicals (Bakulski *et al*., 2012). Five groups of compounds might influence AD: metals, pesticides, industrial pollutants, antimicrobials and air pollutants (Yegambaram *et al*., 2015). Some toxic metals, such as lead and aluminium are related with several neurodegenerative diseases such as AD (Yegambaram *et al*., 2015), showing the role that these environmental pollutants can play in neurodegenerative diseases aetiology. Studies in rodents and primates showed that exposure to Pb in early-life stages could result in neurological changes similar to the changes that occurred in AD in late-life stages (Bakulski *et al*., 2012; Yegambaram *et al*., 2015). Yegambaram *et al*. (2015) hypothesized that the early-life exposures to Pb could increase late-life sensitivity to neurodegeneration and potentially for the development of AD.

These findings indicate a link between environmental exposure and AD and/or PD, two age-related disorders. Since frailty syndrome is also an aged-related disorder with undetermined causal effects, environmental exposures should be considered when studying the potential risk factors of the syndrome.

1.3. Human Exposure Assessment

Humans are daily exposed to a variety of potentially harmful agents (IPCS, 2002). Exposure assessment is a crucial procedure for the identification, evaluation and control of environmental hazards. Human biological assessment is a frequently used approach to provide early warning signals of excessive exposure to substances and for prediction of health risks.

1.3.1. Life-time Exposure Questionnaire

The potential to reduce the prevalence of some major diseases is driving research to understand the totality of exposures over the course of our lifetime. People are daily exposed to various chemicals in a non-occupational way through everyday products frequently used. The chemical manufacturing exists on high production volumes (Calafat *et al.*, 2006) and has been increasing during the last decades. It was projected a 3.4% annual rate growth in production until 2030 (OECD, 2008). Globally, around 8 million chemical substances exist in the industry and there are an increasing number of chemicals to which the health and ecological effects are unknown (Mitchell *et al*., 2013). The assessment of human exposures is difficult since it occurs under non-controlled conditions (Calafat *et al*., 2006). People are daily exposed to various substances successively or even at the same time through various routes – ingestion, dermal contact or inhalation - that could result in different consequences (Klaassen and Amdur, 1996), as the interaction of the different effects (potentiation, synergism, antagonism, additivity) may result in several outcomes.

Occupational exposure due to particular working conditions may play a role in health status (Mehlum *et al*., 2006). Cancer, hearing impairment, skin disease, mental conditions and disorders in diverse systems - respiratory, musculoskeletal, cardiovascular, reproductive, and neurological systems - could be consequences of occupational exposure (Wilson *et al*., 2017).

Exposure history/questionnaire data, environmental monitoring and biomonitoring are the main strategies used to assess exposure (Calafat *et al*., 2006). Taking in account recent data, it is hypothesized that the environment may play a role in frailty development (Garcia-Esquinas and Rodriguez-Artalejo, 2017) thus consequently life-course self-reports/questionnaires about non-occupational and occupational exposures are important when studying about potential environmental-frailty link, even more so in older populations. To our knowledge, this is the first field-study investigating the influence of environmental exposures (past and current) on frailty syndrome status, and also using for that a life-course exposure questionnaire.

1.3.2. Biomarkers

Biomonitoring encompasses the measurement of chemicals, metabolites and adducts providing data despite the route of exposure (Calafat, 2006) allowing the measurement of the internal dose of a compound, the effect of an exposure and assessing individual

susceptibility (Silins and Hogberg, 2011). For this purpose, several biological matrices can be used (urine, fat, nails, hair, breast milk and placenta) but the most common for human biomonitoring are urine, exhaled air and blood (Cockerham and Shane, 1994).

Biomarkers or biological markers are valuable tools in human biomonitoring studies because they are intermediates between exposure and clinical manifestation of the disease, representing the whole continuum from external exposure to effect. However, the detection of a biomarker not necessarily indicates the presence of a disease or toxic process, it may only indicate the exposure of the organism to some xenobiotic (Kamrin, 2003).

Biomarkers are defined as a xenobiotically induced alteration in cellular or biochemical components or processes, structures, or functions that is measurable in a biological system or sample (Klaassen and Amdur, 1996). A good biomarker must be sensitive, specific, relevant, reproducible and measurable in the population. Biomarkers are generally classified into three types, biomarker of exposure, susceptibility and effect (Manno *et al*., 2010; Silins and Hogberg, 2011).

A biomarker of exposure allows a direct measure of body burden, englobing measurement of parent compound, metabolites and protein- or DNA adducts (Gil and Pla, 2001). Their presence only indicates that an exposure has occurred. A biomarker of susceptibility is an indicator of an inherent or acquired limitation of an organism to respond to the challenge of exposure to a specific chemical agent(s) (Silins and Hogberg, 2011). Generally, it is used to identify whether a person or groups of people are susceptible to damage caused by certain chemical or other toxicant. This class of biomarkers serves to describe individual variations in the relationship of biomarkers of exposure and biomarkers of effect. These variations may arise from genetic or non-genetic factors. Genetic factors include polymorphisms that may lead to different individual capacities to activate, detoxify or repair DNA lesions arising from exposure to a chemical. Non-genetic indicators include among others, nutritional status, health status, lifestyle and age.

A biomarker of effect is defined as a measurable biochemical, physiological, behavioural functional or other alteration within an organism that might be elicited by the exposure and depending upon the magnitude can be recognized as associated with an established or possible health impairment or disease (Ainsworth *et al*., 2011; IPCS, 1993). It may indicate alterations on a cellular level and also be predictive of pathological changes in complex disease development serving as an early-sign for deleterious health effects (Silins and Hogberg, 2011). Somatic mutations, changes in tumour-suppressor genes and cytogenetic alterations, such as chromosomal aberrations, micronuclei and aneuploidy are some examples of biomarkers of effect (Greim and Snyder, 2008).

These biomarkers are extensively used tools in human biomonitoring studies, especially in occupational studies to evaluate the genotoxic potential of hazardous chemicals (Ladeira and Viegas, 2016). Cytogenetic alterations, such as chromosomal aberrations and micronuclei are the most used endpoints. The increased frequency of these indicators in exposed workers is a quite consistent finding in most studies (Suspiro and Prista, 2011), including in cancer-patients (Hagmar *et al*., 1998). The use of biomarkers for disease risk assessment increased markedly after the 90s-decade due to its application for evaluate progressive diseases, since symptoms are only manifested long after exposure (Silins and Hogberg, 2011). Some authors state that biomarkers are generally more accurate and sensitive than most clinical tests and consequently allow more efficient associations between health impairment and chemical exposure in workers with initial alterations (Ladeira and Viegas, 2016). Although, it should be noted that these biomarkers are not chemical specific, and therefore the association with an exposure must be established by an independent measure, such as environmental monitoring or simultaneous measurement of a biomarker of exposure. Nonetheless, increases on the levels of these endpoints in a group of subjects compared to controls are a clear indication of chromosomal or genomic instability.

Genomic instability refers to a set of genetic events able to cause unscheduled genomic alterations, temporal or permanent (Aguilera and Gomez-Gonzalez, 2008). Most age-related diseases and ageing signs are the consequences of genomic instability and DNA damage, and also a number of them are related to alterations in endocrine and immune systems. Thus, genomic instability might be, directly or indirectly, a primary cause in the ageing phenotype (Coppede and Migliore, 2010).

Ageing mechanisms are far to be understood and although chronological age is the most widely criteria used to evaluate ageing process, it cannot explain the differences that occur in people of the same age (Martin-Ruiz and von Zglinicki, 2014). The differences observed in some studies in health and functional status between individuals even with the same age group (Collard *et al*., 2012) indicate that some individuals are more prone to develop some characteristics than others. The difference of age between the first and the last individual dying in a cohort study is usually greater than the mean and median lifespan in the cohort (Martin-Ruiz and von Zglinicki, 2014). In this context, it is difficult to define ageing since the process occurs in a distinct way on individuals, and consequently, the term “biological age” or “biological ageing” appeared and was defined by Martin-Ruiz and von Zglinicki (2014) as an “ever-increasing intrinsic probability of death with progressing time”.

Although it is generally recognized to have a biological basis, no particular biological trait has been consistently associated with frailty status so far (Fougere *et al*., 2015). Biological basis of frailty includes three dimensions: cellular level, systematic level and organismic level (Sanchez-Flores *et al.,* 2017) (Figure 4). Some investigation is being conducted, namely at endocrine and immunological level, with inconclusive results (Puts *et al*., 2005a; Fernandez-Garrido *et al*., 2014;). Variations of oxidative stress biomarkers were associated to frailty status in older people (Fougere *et al*., 2015), while for genomic instability this association was not clear (Myers *et al*., 2013). Recent studies have found positive correlation between frailty status and DNA methylation, linking this syndrome to epigenetic alterations. Several pathophysiological mechanisms of frailty remain uncertain (Collerton *et al*., 2012). Pro-inflammatory status, hypothalamic-pituitary axis function alterations, sympathetic nervous system activity enhanced and abnormal renin-angiotensin system stimulation have been hypothesized as cellular alterations that lead to frailty phenotype (Fielding, 2015). These systemic alterations could ultimately be linked with apoptosis, senescence, epigenetic modifications and other mutations (Fielding, 2015). Although the studies in this field are still limited the established link between DNA repair, genomic instability, and age and age-related disorders encourage deeper investigations on this line (Sanchez-Flores *et al.,* 2017).

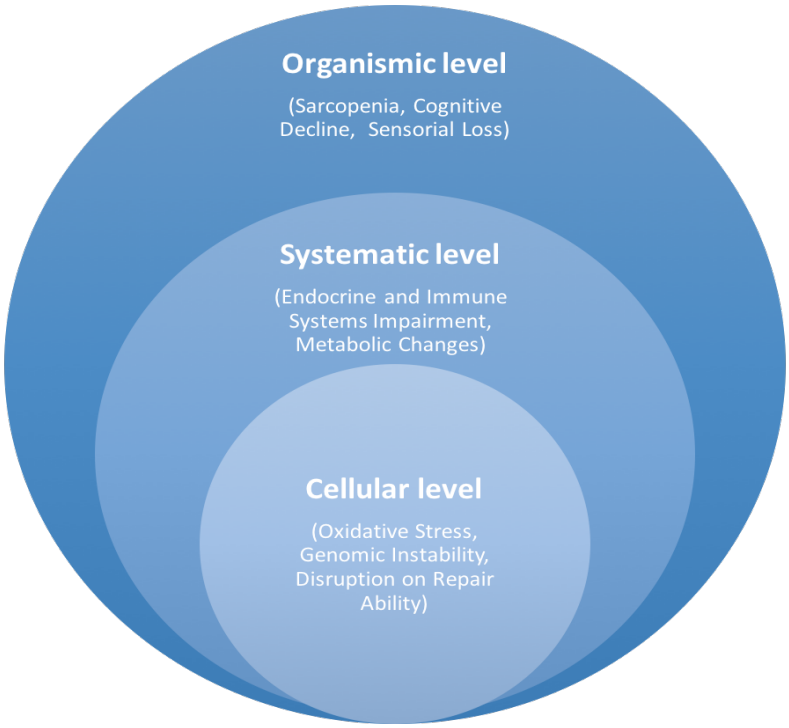

Figure 4: Biological basis of frailty. Adapted from Sanchez-Flores *et al.,* 2017.

1.3.2.1 Comet assay

The single cell gel electrophoresis assay, generally known as comet assay, has been proven to be a very sensitive tool in human biomonitoring for the detection of different levels of DNA damage at the individual cell level (Collins, 2004). It is based on the ability of negative charged fragments of DNA to be pulled through an agarose gel in response to an electric field, appearing like a "comet".

The main advantages of the comet assay include: a) sensitivity for detecting low levels of damage, b) single cell data collection, allowing more robust statistical analyses, c) requirement for a small number of cells per sample, d) low cost, e) rapid and ease of application and f) flexibility to use fresh or frozen samples (Costa and Teixeira, 2014; Glei *et al*., 2016). Furthermore, comet assay can be used in any eukaryote monodispersed cell population, proliferating as well as nonproliferating including, cultured mammalian cells, peripheral blood mononuclear cells, disaggregated tissues biopsies, haemolymph from molluscs, yeast, and nuclei isolated from plant tissue demonstrating its vast matrix appliance (Collins, 2014). For most purposes, however, well-characterised cell lines or primary cells (e.g. peripheral blood cells) used in classical genetic toxicology testing assays, are preferred.

Comet assay principle was first introduced in 1984 by Ostling and Johanson, based on previous work published in late 1970s by P. Cook. Cook and colleagues developed a method to investigate nuclear structure of eukaryotic cells based on high salt lysis with non-ionic detergents. The single cell gel electrophoresis approach by Ostling and Johanson resulted in structures resembling comets, with a distinct head, comprising intact DNA and a tail of extended DNA loops and DNA fragments. As a consequence, the name "comet assay" was given (Piperakis, 2009).

Several versions of the comet assay are currently in use being the most popular the alkaline version, first introduced by Singh *et al*. (1988) (Costa and Teixeira, 2014). Briefly, cells are embedded in agarose, placed on a microscope slide and lysed with detergent at high salt concentration. During lysis, cellular membranes, cytoplasmic and nucleosplasmic constituents and most histones are removed. After lysis, what remains is the nucleoid containing RNA, proteins and negatively supercoiled DNA attached to the nuclear matrix. The immobilised nucleoid is then denaturated in an alkaline buffer and electrophoresed in same buffer. The "comet" can be visualised with a fluorescence microscope after staining with a DNA staining dye (e.g: SYBR®Gold, ethydium bromide, acridine orange). The size

and shape of the comet and the distribution of DNA within the comet correlates with the extent of DNA damage present in the individual cell.

In order to improve comet assay's sensitivity and selectivity, Azqueta *et al.*, (2009) introduced an extra step after lysis: incubation with lesion-specific enzymes that are used to convert DNA lesions to DNA breaks, such as oxidized bases. Thus, different enzymes can be used to estimate the levels of oxidative damage, namely formamidopyrimidine glycosylase (FPG), that detects oxidized purines (e.g: 8-oxoguanine) as well as other lesions; hOGG1, identifies 8-oxoguanine; endonuclease III (EndoIII), reveals oxidized pyrimidines; T4 endonuclease V, exposes UV induced cyclobutane pyrimidine dimmers and uracil glycosylase (UDG), that recognize uracil bases in the DNA sequence. These enzymes are DNA repair glycosylases that slide along the DNA chain, recognize damaged bases and cut the glycosidic bond between the damaged base and the sugar of DNA structure (Friedman and Stivers, 2010).The resulting abasic site leads to a strand break either by the enzyme's associated lyse activity or by the subsequent alkaline treatment (Ersson, 2011).

Incubation of cells with only buffer or buffer containing enzyme allows an estimation of both DNA breaks and the calculation of net enzyme-sensitive sites. The most frequently used enzyme is the FPG which recognise oxidised purines, namely 8-oxoGua, but also other lesions (Azqueta *et al*, 2009).

Comets can be analysed by visual scoring, semi-automatic computerised image analysis or by fully automatic computerised image analysis (Collins, 2004). In visual scoring comets are divided into five categories (from class 0 to 4) representing increasing relative tail intensities and therefore increasing damaged cells (Collins, 2004). Computerised scoring is reported using a range of different parameters. The most frequently used endpoints are percentage DNA in tail (%TDNA), tail moment and tail length. The different ways of scoring cells have different advantages and limitations. Azqueta *et al*. (2011) recently compared visual scoring, computerised scoring and automatic computerised scoring and concluded that all three scoring methods are reliable and the results from the three methods are, to a certain extent, comparable.

During the last decade, several approaches have been used to turn this method faster by analysing more samples in the same experiment (Shaposhnikov *et al.,* 2010) or effective by eliminating some preliminary procedures (Al-Salmani *et al*., 2011). One of the medium throughput method that allows to analyse more samples is the 12 mini-gels set in a slide. In this method, a device that clamps a gasket and the slide to a metal support is used, creating individual wells that can be treated separately. For most of the human genotoxicity

assays lymphocytes are the preferred cells, since they are sentinel cells that may inform about events occurring in target tissues (Costa *et al*., 2015). However, the use of whole blood as a viable and easier alternative in comet assay has been recently studied by several authors (Akor-Dewu *et al*., 2014). The main advantages presented is to avoid added DNA damage from lymphocyte-isolation steps and loss of cells (Al-Salmani *et al.,* 2011; Collins *et al.,* 2014).

1.4. Frailty – Prevention

Frailty syndrome in older adults is an important concern in society since frail individuals have special needs that require specialized medical care (Collard *et al*., 2012). Even more, frailty may be reversed at a certain stage (Ahmed *et al*., 2007; Morley *et al*., 2013) and some treatment can be effective either by reverting or preventing the development of this debilitating syndrome. The most frequent situation is the progression of a minor status of frailty to upper frailty stages, but the contrary situation was also reported (Wilson *et al*., 2017). Physical activity practice, caloric and protein supplementation, vitamin D, support/exposure and reduction of polypharmacy are some of the possible measures that should retreat frailty syndrome. Moreover, since weight loss and obesity are related with frailty, in many cases an adequate nutritional supplement, based in caloric and right protein levels adapted for each person could help to depict these factors. Weakness, demonstrated by diminished strength, could also be regulated by physical exercise or physical activities (Morley *et al*., 2013). Deficit of vitamin D in elderly was associated with some events, such as falls, hip fractures and even mortality, and consequently is common the supplementation of vitamin D with the aim of trying to revert it (Morley *et al.,* 2013). Some authors support that the main targets for treatment of frailty are sarcopenia and chronic malnutrition (Clegg and Young, 2011), while others considered that polypharmacy could be a major contributor for frailty when unappropriated medicines are used, highlighting non-prescription medicaments or supplements as the major concern (Morley *et al*., 2013). Frailty is a multifactorial syndrome that includes different types of diseases, and for this reason, it is still unknown if all possible treatments settle all individuals that manifest with frailty (Rodriguez-Artalejo and Rodriguez-Manas, 2014).

Bio-gerontology has the goal of defining biological markers of ageing more predictable than chronological age to indicate the biological age of populations, groups and individuals (Martin-Ruiz and von Zglinicki, 2014). Since biomarkers could be an intermediate between exposure and clinical manifestation of the disease as stated above, the establishment of an

efficient biomarker of frailty could help to set the causes of frailty syndrome and consequently to define the measures to be taken for frailty prevention.

AIM OF STUDY

The frailty syndrome in older adults is already a problem faced nowadays and a current priority theme of discussion in European Union (Healthy) Ageing agenda. The elder population (above 65 years old) is growing promoting an accentuated ageing scenario in the near future. Frailty has been demonstrated to be the most common condition leading to disability, institutionalization and death in the elderly. Therefore, knowledge about the frailty status of a subject before the development of clinical symptoms is crucial, even more if considering that frailty is seen as being potentially reversible. In this context, prevention on frailty may help to plan interventions (pharmacological and not), stop decline in physical function and disability, as well as in organizing the healthcare systems and their impact on patient outcomes hence reducing the socio-economic costs.

Given the serious consequences of frailty is essential to understand why/how some older adults in same age-range become frail while others do not. Currently, there are several gaps in scientific literature regarding frailty syndrome. In this context, is necessary to deepen the knowledge about frailty syndrome and its aetiology, for consequently prevent it. Taken in account the multifactorial nature of this syndrome, studies should also gathered different areas namely public health, environmental toxicology and social sciences.

The main goal of the present work was to contribute for the knowledge of frailty syndrome in older adults namely investigate potential risk factors and mechanistic pathways of frailty syndrome. Exposure was assessed via a life-course questionnaire allowing the evaluation of current and previous environmental (including occupational) exposures that potentially may be associated to development of frailty syndrome.

For this purpose, the main objectives of this study were:

I. To build and apply a life-course exposure history questionnaire in an elderly population of Oporto metropolitan area. The lifetime exposure questionnaire provides information about various periods of life, jobs/activities, neighbouring industries and indoor and outdoor air pollution;

II. To apply a frailty questionnaire and classify the individuals about their frailty status, as frail, pre-frail, or robust;

III. To measure DNA basal damage and oxidative damage levels of individuals using single cell gel electrophoresis assay;

IV. To cross and relate data with the aim of understanding the relationship between exposure – frailty syndrome – DNA basal/oxidative damage.

This study is part of a larger project engaging different institutions, the *Biofrail Project* and it constitutes the first report about the studied population.

MATERIALS AND METHODS

1. Study Population

Community institutions (senior associations, day care centres, elderly care centres) located in the metropolitan region of Oporto were contacted to participate in the study. Meetings were scheduled with the main objective to seek institutional approval and authorisation to contact the target subjects.

Selected subjects (and their families or proxies, if required) were informed on the nature of the study, principal aims, nature of participation (what is required), risks and benefits. The participation in the study was voluntary (no financial compensation was offered) and stopped if requested at any point. Leaflets with this information were distributed. Eligible for inclusion in the study were a) subjects with 65 years old or over living at home or in retirement homes (private or state). The exclusion criteria included: a) inability to stand and walk independently, b) severe dementia and/or cognitive impairment, c) lack of ability to communicate, d) severe impairment of sight and hearing and e) receiving palliative care.

Between October 2016 and May 2017, 68 subjects from senior recreational community associations and day care centres were recruited for this study. The institutions that agreed to participate (n=3) had a universe of 121 subjects, 68 accepted to participate in this study, the response rate was around 56%. Of these 68 participants, seven subjects were excluded: five participants had less than 65 years and two quit the study due to disease questions and lack of available time to answer the questionnaire. After exclusions and withdrawals, a final number of 61 participants was established. Each person was asked to sign the informed consent form before being enrolled in the project in compliance with Declaration of Helsinki and Oviedo Convention. All personal data was stored separately from participant's name and other identification marks that could unambiguously identify the user. Samples and data of each subject were identified only by a unique code number. Anonymous databases were established with the experimental results obtained along the investigation process. Researchers only manage these secondary databases for uploading the experimental data generated and for further statistical analysis.

2. Questionnaires

Two questionnaires were applied to all individuals – a frailty questionnaire and a lifetime exposure questionnaire. In addition, subjects were also inquired on general medical history, medication, diagnostic tests (X-rays, etc.) and other relevant individual information such as

age, smoking habits and alcohol consumption. The questionnaires were coded to ensure participants anonymity and confidentiality.

2.1. Frailty Questionnaire

Frailty questionnaire was applied to all individuals. The purpose of the questionnaire was to evaluate the frailty status of each subject through five criteria of frailty as proposed by Fried *et al*. (2001) weakness, slowness, low physical activity, exhaustion and weight loss. Following the author and the cut-offs established 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 scores more than three criteria, pre-frail, if score one or two criteria, or robust, with zero criterion scored (Fried *et al*., 2001). All criteria were assessed according to Fried *et al.* (2001), as described in the following sections, with some minor modifications on the physical activity evaluation (2.1.3).

2.1.1 Weakness

Weakness was evaluated through grip strength using a hydraulic hand dynamometer *JAMAR*® (5030J1). For each participant three measurements were recorded with both hands, but only the average value of the measurements obtain with the dominant hand was used. For the assessment, the participant was asked to seat in a chair, with the back straight, the elbow flexed to 90º and forearm and wrist in neutral position. The participant was requested to squeeze the dynamometer with his maximum strength. The cut-offs for this parameter dependent on gender and BMI quartiles, accordingly to Fried *et al*. (2001), are described in Table I.

Table. I: Cut-offs for weakness defined by Fried *et al.* (2001).

	Men				**Women**			
BMI	≤24	24.1-26	26.1-28	> 28	≤23	23.1-26	26.1-29	> 29
Cut-off for frailty	≤29	≤30	≤30	≤32	≤17	≤17.3	≤18	≤21

2.1.2 Slowness

For slowness assessment, it was asked to each participant to walk 4.57 meters in a straight line at a comfortable pace and fast speed. For this task, a digital chronometer *GEONAUTE (On Start 100 Stopwatch)* with 1/100th second and one split time was used. Each participant performed the trial three times and the speed was registered in minutes, seconds and milliseconds. The shortest time performed was used to classify frailty for this criterion, the cut-offs (dependent on gender and height) used were applied according to Table II.

Table. II: Cut-offs for slowness defined by Fried *et al.* (2001).

	Men		**Women**	
Height	≤173 cm	>173 cm	≤159 cm	>159 cm
Cut-off for frailty	≥7sec	≥6 sec	≥7 sec	≥6 sec

2.1.3 Low Physical Activity

Low physical activity assessment was carried out according to an adapted Version of Spanish Validated Short Version (VREM) (Ruiz Comellas *et al*., 2012) of Minnesota Leisure Time Physical Activity Questionnaire (MLTPAQ) (Taylor *et al*., 1978). A validated instrument for assessing the elderly physical activity criteria is missing in Portugal, a questionnaire designed for the whole population is not suitable for older groups. Currently, in Portuguese studies on frailty this criterion is evaluated by one dichotomous question (Do you practice physical activity?) (Coelho *et al*., 2015; Duarte, 2013).

Fried *et al.* (2001) used a self-reported questionnaire for assessing physical activity adapted from the MLTPAQ (1978 version). The MLTPAQ is a questionnaire built by Taylor *et al.* (1978) where physical activities were related with metabolic equivalents (METs), in which a MET corresponds to one kilocalorie per kilogram of body weight per hour (kcal/kg/h) or to 3.5 millilitres of oxygen per kilogram of body weight per minute (mL O_2/kg/min).

This MLTPAQ version does not comply with our study population since most of the physical activities assessed (e.g. playing tennis, golf) are not similar with those performed by the overall Portuguese elderly population. Furthermore, the MLTPAQ version used by Fried *et al*. (2001) was not validated for elderly subjects, but for the general population. Recently Ruiz Comellas *et al*. (2012) have validated a “Spanish Short Version of Minnesota Leisure Time Physical Activity Questionnaire (VREM)”. An adapted version of the questionnaire

used by Fried *et al.,* (2001) with lower number of criteria in order to reduce the time of questionnaire and to adapt the physical activities to Spanish elderly population (Ruiz Comellas *et al*., 2012). Similarly, with MLTPAQ in VREM to each physical activity corresponds a MET value, listed on the Compendium of Physical Activities (Ainsworth *et al.,* 2011). The physical activity is calculated through the energy expenditure in 14 days in METs by minute (METS-min/14days) (Ruiz Comellas *et al*., 2012). At the end, individuals are classified according to their energy expenditure: sedentary individuals if had less than 1,250 METS-min/14days; moderately active if had between 1,250 and 2,999 METS-min/14days; active if had between 3,000 and 4,999 METS-min/14days; and very active if had more than 5,000 METS-min/14days.

VREM questionnaire presents some advantages when compared with the adapted version of MLTPAQ used by Fried *et al.* (2001) namely the short time required; ease of application and interpretation and; the questionnaire being addressed to elderly population, especially to the Spanish elderly population culturally similar to the Portuguese (Ruiz Comellas *et al.,* 2012).

Table. III: Cut-offs defined for physical activity defined to Fried *et al.* (1).

	Women	**Men**
Cut-off for frailty	< 270 kcal/week	< 283 kcal/week

2.1.4 Exhaustion

Following Fried *et al.* (2001) exhaustion was measured through two questions of the CES-D (Center for Epidemiological Studies Depression Scale): “How often in last week did you feel that everything you done was an effort?” and “How often in last week did you felt lack of energy?”. The answers were scored as follows: zero points for “rarely or none”, one point for “some or a little of the time”, two points for “a moderate amount of the time” or three points to “most of the time or always”. In order to classify this parameter, it was considered that the participant was frail for exhaustion when the answer given scored two or three points to either of these questions.

2.1.5. Weight Loss

Weight loss was self-reported accordingly to Fried *et al.* (2001) with modifications, since the original question was in pounds, 10 pounds corresponding to 4.5 kg. The question “In the

last year, did you lost weight unintentionally (i.e., not due to dieting or exercise)?" was made to all participants, followed of "How much do you think you lost?". The answer "yes" to this question, i.e. when the participants had lost more than 4 kg, defined a frail status for weight loss criteria.

2.1.6. Body Mass Index (BMI)

Anthropometric measurements for each participant were obtained during frailty questionnaire and biologic sample collection. Weight was recorded using a SECA® 761 flat mechanic scale to the nearest 0.5 kg (SECA GMBH & Co. Kg., Hamburg, Germany). Height was recorded to the nearest millimetre using a portable stadiometer SECA® 213 (SECA GMBH & Co. Kg., Hamburg, Germany) was registered for the calculation of BMI.

2.2 . Lifetime Exposure Questionnaire

Lifetime Exposure Questionnaire (LTEQ) is a tool developed in this study with the aim to assess individual relevant exposures to contaminants that could have any role or influence in elderly health.

3. Comet Assay

3.1. Blood sample collection

Venous blood samples were collected in 6-ml Vacutainer® EDTA (ethylenediamine tetra-acetic acid) tubes (Becton, Dickinson and Company) by venipuncture from an antecubital vein during the morning. Blood was immediately stored at 4˚C, transported (within 30 min maximum) to the laboratory and immediately processed for further analysis. All samples were coded and analysed under blinded conditions. Blood for comet assay analysis was cryopreserved. For cryopreservation, an equal amount of 1:4 (V/V) mixture of DMSO (dimethyl sulfoxide) and RPMI 1640 was added to blood samples. Samples were then stored in aliquots (200 µl each) at -80˚C. Before the assay, the frozen blood samples were rapidly thawed at room temperature and washed twice (centrifugation at 223g for 10 min) with 5 ml of PBS.

3.2. Alkaline Comet Assay

The alkaline comet assay was performed as described by Singh *et al*., (1988) with minor modifications (Costa *et al*., 2008). A medium-throughput version of the comet assay 12-Gel Comet Assay Unit ™ (Severn Biotech Ltd) was used. 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 for each subject in three slides (2x 3 slides); one slide to assess basal DNA damage and two slides to evaluate oxidized purines through enzymatic repair activity. Gels were allowed to set for 2-5 min at 4ºC 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 4ºC. Slides were placed on a horizontal electrophoresis tank, covered with cold alkaline electrophoresis solution (1mM Na_2EDTA, 300mM NaOH, pH 13) and left for 40 min in the dark to allow DNA unwinding. Electrophoresis was carried out for 20 min at approximately 1.2 V/cm. Subsequently, slides were washed in 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 µL 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 utilized 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 used to describe comet formation.

3.3. Enzyme-Comet Assay

The comet assay enzyme version was performed as described by Azqueta and Collins (2013) (Figure 5). FPG (formamidopyrimidine DNA glycosylase) was the enzyme selected to measure the amount of DNA oxidized purines. Briefly, after lysis, slides for enzyme 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 (3.2). Net FPG-sensitive sites were calculated by subtracting the %TDNA values for the slide incubated with buffer from the score for the slide incubated with the enzyme.

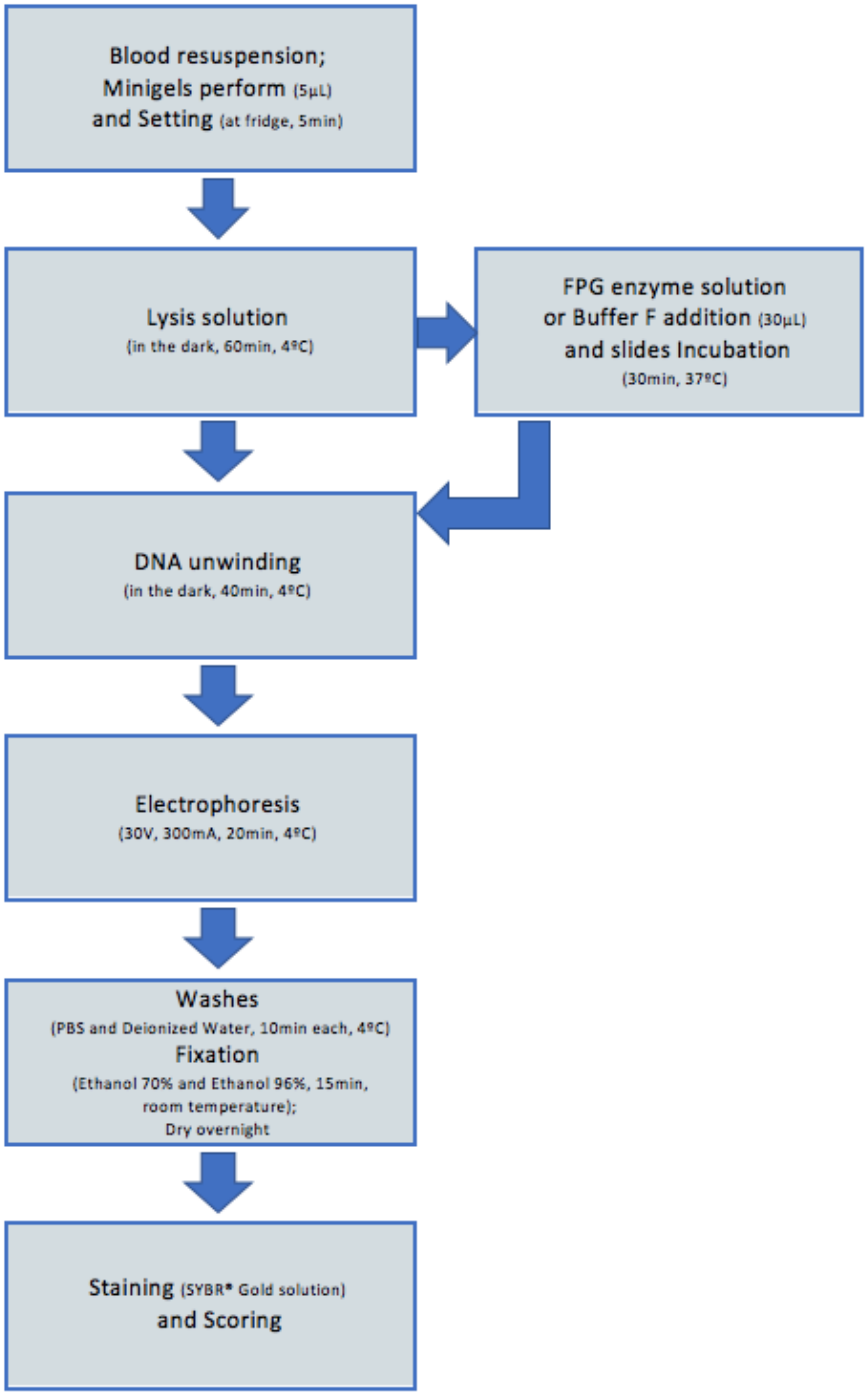

Figure. 5: Diagram of Alkaline Comet Assay Version and Enzyme-Comet Assay procedure.

4. Statistical Analysis

The distribution of variables was compared with the normal distribution by means of the Kolmogorov–Smirnov goodness-of-fit test. The oxidative DNA damage parameter (Net-FPG) was the only parameter that departed significantly from normality, no transformation was done.

A general description of the study population was performed through univariate analysis. The distribution within the study groups of socio-demographic, lifestyle factors and exposure-related factors was evaluated with the Student's t-test for continuous variables and the Pearson's Chi-square test for categorical variables. Non-parametric tests Mann–Whitney U-test and Kruskal–Wallis test were applied to continuous variables not following normal distribution. Associations between variables were tested by Pearson's correlation coefficient (for parametric variables) or Spearman's rank correlation (for non-parametric variables). The level of statistical significance was set at 0.05. All analyses were performed using the IBM SPSS Statistics V. 24.0 software for Windows.

RESULTS

1. Study Population

The general characteristics of study population and study groups (robust, pre-frail and frail) are summarized in Table IV. Possible differences between groups regarding several variables were assessed. Since only two individuals were classified as frail, in agreement with Fried *et al.* (2001) criteria, only robust and pre-frail groups were considered for statistical testing.

Table IV. Characteristics of the study population and frailty groups (n=61).

	All (n=61)	**Robust (n=29)**	**Pre-Frail (n=30)**	*p-value*	**Frail (n=2)**
Gender					
Females	40 (65.6%)	15 (51.7%)	23 (76.7%)	$p<0.05$[b]	2
Males	21 (34.4%)	14 (48.3%)	7 (23.3%)		-
Age (years) [a]	77.3±7.1	74.8±1.1	79.2±1.3	$p<0.05$[c]	88.5±6.5
Smoking Habits					
Never smokers	44 (72.1%)	19 (65.5%)	23 (76.7%)	$p>0.05$[b]	2
Ex-smokers	17 (27.9%)	10 (34.5%)	7 (23.3%)	$p>0.05$[b]	-
Years smoking [a]	21.5 ± 5.5	21.5±3.7	24.6±6.8	$p>0.05$[c]	-
Years as ex-smokers [a]	32.3±3.81	34.5±4.1	29.3±7.5	$p>0.05$[c]	-
Body Mass Index (BMI)	28.5±0.5	28.2±0.6	29.1±0.8	$p>0.05$[c]	24.6±4.2
Underweight (< 18.5)	-	-	-		-
Normal Weight (18.5 – 24.9)	12 (19.7%)	5 (17.2%)	6 (20%)		1
Overweight (25.0 – 29.9)	31 (50.8%)	17 (58.7%)	13 (43.3%)		1
Obese (≥30)	18 (29.5%)	7 (24.1%)	11 (36.7)		-
Medication use [a]	5.1±0.4	4.8±0.5	5.2±0.5	$p>0.05$[c]	5.0±4.0

[a] Mean± standard deviation; [b]Chi-square test; [c]*t*- Student test

A significant difference was found regarding gender and age in robust and pre-frail group. The study population was composed mainly by women (65.6%). The pre-frail group had 76.7% (n=23) while robust group included 51.7% (n=15) of women. Furthermore, the pre-frail group had half the men compared to robust group. Frail group (n=2) consisted only of women.

Considering age, the pre-frail group was significantly older than the robust (79.2 ±1.3 *vs* 74.8 ±1.1), with around four years of difference. The mean age of the frail group was 88.5 ±6.5 years old. The distribution of the study population stratified by age according to their frailty status is shown in Figure 6. Overall, the robust phenotype is displaced to the younger age classes, with a peak of frequency between 71-75 years old, compared with the pre-frail phenotype that has a constant distribution along most classes but has a peak in subjects aged between 81-85 years old. Although frail group is only constituted by two individuals, it

is possible to see that in this study population the frail phenotype was manifested in individuals over 80 years old.

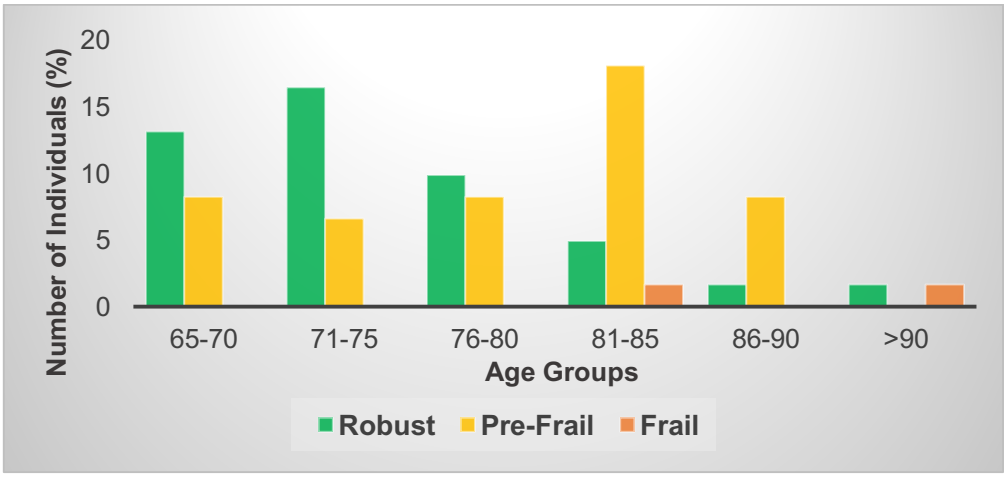

Figure 6. Distribution of the study population by age groups and frailty status.

Smoking habits groups were established as never smokers and ex-smokers (subjects that stop smoking for at least 2 years), since there were no current smokers reported in the study population (all were non-smokers). The average years as ex-smokers were higher than 30 years. No differences were found between robust and pre-frail groups concerning the smoking habits. Frail individuals stated to be never smokers.

A significant difference was found between the number of second-hand smokers in robust and pre-frail groups; 23.3 % of the pre-frail subjects reported to be second-hand smokers while among robust subjects this fraction was 10.4%.

No differences were found regarding the body mass index, the mean average was 28.5±0.5.

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. In our study population, there were no differences between groups regarding the daily intake of medication, an average of 5 drugs/day by individual was found.

The number of medication ranged between one to twelve different drugs, all participants reported the intake of medication. In Figure 7 is represented the data regarding the number of daily medication of the study population, showing the quantity of medicines used per day. Two participants of the study do not answer the number of drugs used by day, since they did not know that information.

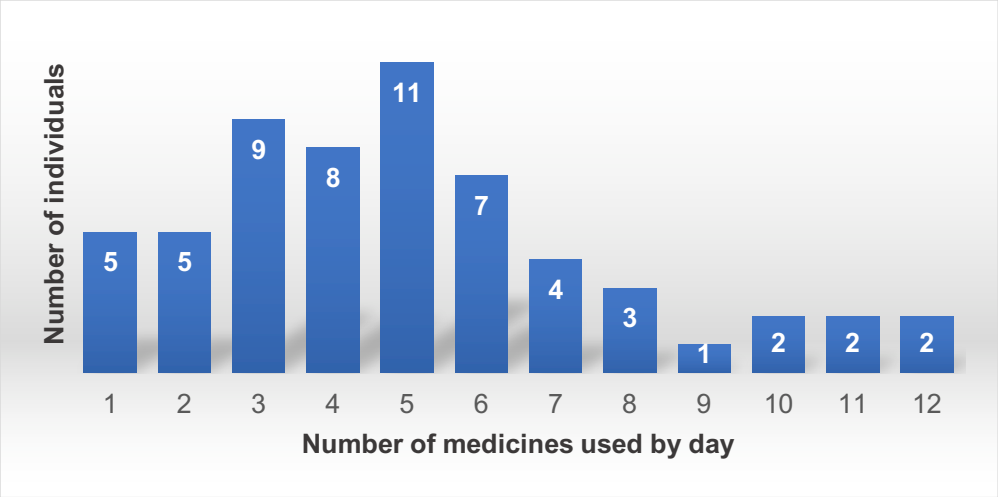

Figure 7: Number of medicines used daily by the study population

2. Frailty Questionnaire

Frailty status was assessed using the Fried's Frailty Model with minor adaptations, as described in Materials and Methods section (2.1).

2.1 Frailty Criteria

The distribution of frailty phenotypes in the study population is represented in Figure 8. Of the total population, 47.5% (n=29) were classified as robust, 49.2% (n=30) were categorised as pre-frail and 3.3% (n=2) were considered frail.

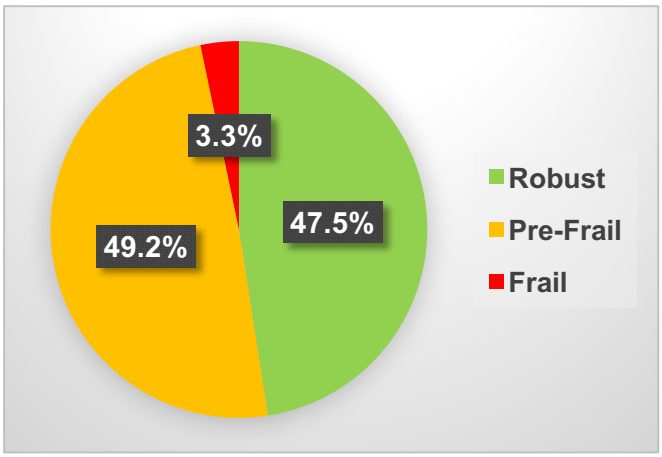

Figure 8: Prevalence of frailty syndrome in the study population (%).

The Figure 9 represents the absence and presence of the five frailty criteria in the study population, following the cut-offs established by Fried *et al*. (2001). Each component of frailty was punctuated with "zero" or "one", according to its absence or presence, respectively.

The frequencies obtained for each component of frailty revealed that weakness, walking speed and exhaustion criteria were the parameters contributing more for the classification of frail phenotypes (frail and pre-frail) in our study population. Weight loss was scored by 8.2% (n=5) of the individuals studied. Weakness was the criteria more prevalent, 32.8% (n=20), following by exhaustion, 26.2% (n=16), slowness, 11.5% (n=7), and lastly weight loss. No individual scored positive for low physical activity, hence, all individuals in this criterion were punctuated as "zero" and considered "fit".

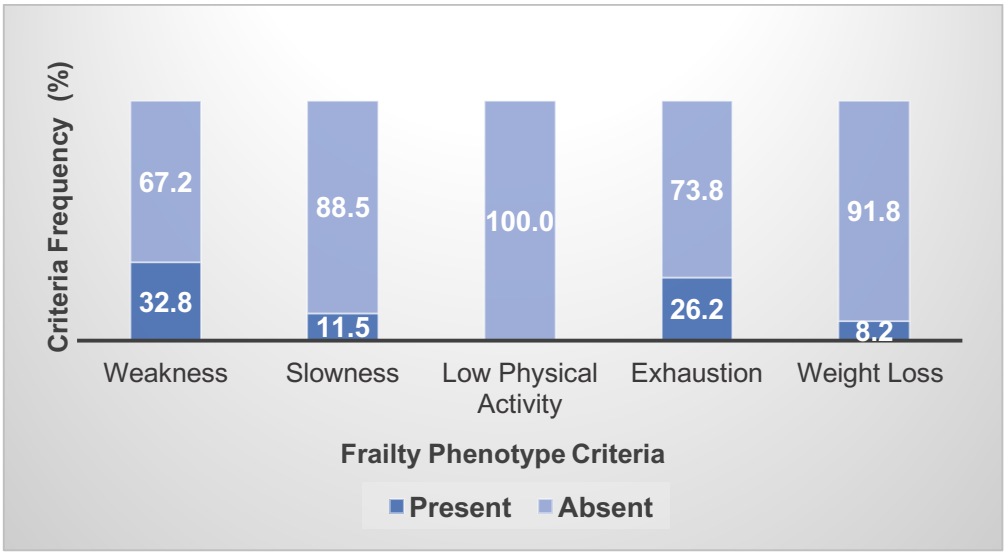

Figure 9: Prevalence of each frailty criteria in the study population.

2.2 Frailty phenotype assessment

Among the 61 participants of the study, 29 were considered robust (negative for all five criteria) and 32 were classified either pre-frail, the transitory state of the syndrome (n=30; positive for one or two criteria) or frail (n=2; positive for three or more criteria). None of the participants were positive scored for four or five criteria. In the Figure 10 is shown the frequency of the number of criteria scored positively for frailty status, only pre- frail and frail subjects were considered. Regarding the results, of the 32 individuals 56.3% scored one criterion, 37.5% scored two criteria, and 6.3% scored three criteria.

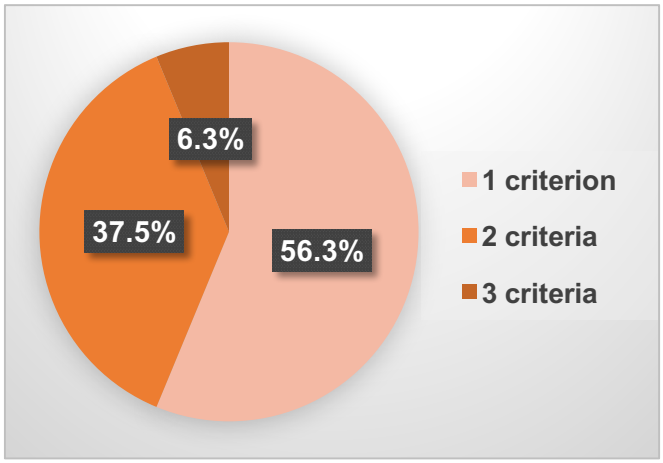

Figure 10: Number of criteria present in individuals in pre-frailty or frailty (n=32)

Considering each group individually, among the pre-frail subjects (n=30), 60.0% displayed one criterion and 40.0% accounted for two, as for frail subjects (n=2), all were positive for three criteria, thus, no frail individual was classified for the presence of four or five criteria. Some criteria have contributed more for the classification of the syndrome than others. The contribution of each frailty criteria for the prevalence of pre-frail individuals in the study population are represented in the Figure 11. The results obtained were similar to those observed in Figure 8, weakness was the component that most contributed for classifying individuals as pre-frail (60.0%) followed by exhaustion (46.7%). Weight loss and slowness contributed equality (16. 7%) for this phenotype classification.

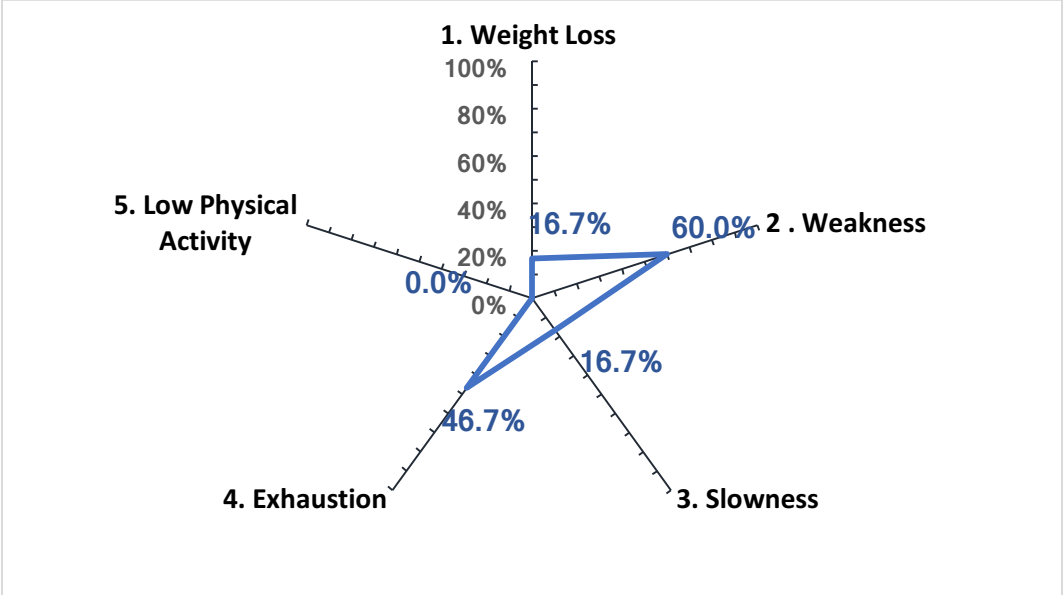

Figure 11. Contribution of each component of frailty for prevalence of pre-frailty (n=30)

In our study population, weakness, slowness and exhaustion were the criteria that contributed to classified individuals as frail. No weight loss or low physical activity was found.

3. Exposure Questionnaire

Life-time course exposure questionnaire (LTEQ) was applied to study population (n=61), all 61 questionnaires were considered valid. All the variables of the LTEQ were associated with the presence or absence of a risk of environmental (or occupational) exposure to contaminants.

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 were considered a potential risk of environmental exposure to pollutants (e.g. particulate matter, hazard chemicals). Similarly, the presence of sources of local pollution near home namely farms (agricultural production), livestock farming, slaughterhouses, landfills, mines, industrial production areas and/or traffic zone represent a possibility of environmental exposure to several air and water contaminants. Regarding diet, 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 the regard to the use of pesticides, sulphate or other agricultural-related compounds.

In the study population, no particular exposure-parameter, current or past, were significantly associated to either robust or pre-frail phenotype, with the exception of the consumption of vegetables home-produced, in private farmhouses. More robust individuals reported to consume vegetables from this source compared to pre-frail individuals.

Household proximity to traffic (road, rail and sea) was also assessed, both current and past. All participants reporting living near a traffic zone (90.2%), selected road traffic as the type of traffic. The level of exposure was categorised according to the intensity of traffic reported

by participants (multiple choice question) as: not intense (low intensity), intense and highly intense. Only 9.8% of the participants reported not living near a traffic area. Nevertheless, no significant differences were found in the number of subjects classified as robust or pre-frail living in those areas. Similar results were found concerning road traffic nearby past households.

Regarding occupational exposure, it was asked the history of past professions and major tasks performed. Participants also gave information about potential occupational exposure to solvents, particulate matter, chemicals and radiation, frequency of exposure and PPE usage. This information was matched and linked to an occupational exposure matrix which allowed to categorise the probability of exposure to “pesticides”, “organic solvents” and “metals” as unlikely, possible and likely to occur. Considering, all population, occupational exposure to organic solvents was possible to occur while exposure to pesticides and metals on work settings were unlikely to occur. No significant differences were found associating the likelihood of exposure to the three contaminants and the frail phenotypes analysed, robust and pre-frail.

DISCUSSION

In the last years, frailty syndrome in older adults became a theme of growing importance due to: a) it is a frequent condition, with high prevalence observed in several studies (Fries *et al.*, 2011), b) its ability to be prevented, delayed or even reversed (Lang *et al.,* 2009), and c) be the most common condition leading to disability, institutionalization and death in older adults (Clegg *et al.,* 2013). The prevention of frailty in old age is one of the key actions identified in Horizon 2020 Framework Program in the context of (Healthy) Ageing (Rodriguez-Artalejo and Rodriguez-Manas, 2014).

Despite this syndrome may occur in younger populations under certain circumstances (e.g. myocardial infarction), frailty is intimately associated with older elderly population (Walston *et al.*, 2006).

Thus, it is expected that the prevalence of frailty will follow the ageing tendency in the next years and will display higher rates (Ahmed *et al*., 2007). In an attempt to revert this scenario several studies have been focused in deepen the knowledge about frailty (Rodriguez-Artalejo and Rodriguez-Manas, 2014).

In the present work, the effect of environmental and occupational exposures during the life-time course and its relationship with frailty prevalence in a population aged 65 years and over was studied. Frailty was assessed using the Fried's Frailty Model with few adaptations for low physical activity criteria measurement. Furthermore, the basal damage and the oxidative damage of individuals was assessed through the comet assay technique.

The present study population (n=61) was classified as being 47.5% robust, 49.2% pre-frail and 3.3% frail.

In a cohort comprising 5317 older adults Fried *et al.* (2001) reported a prevalence of 46.4% robust, 46.6% of pre-frail and 6.9% of frail subjects. This data is consistent with those found in the present study: a low prevalence of frailty, and a similar prevalence of non-frailty (robust) and pre-frailty; with pre-frailty status displaying a slight increase compare to non-frailty. In a systematic review by Collard and colleagues (2012) a wide variation in the prevalence of frailty status was reported ranging from 4.0% to 59.1%. Studies included in Collard's review assessed frailty using different methods including self-report screening instruments, yielding high rates of frailty prevalence. When only the studies using Fried's Frailty Model were analysed the prevalence of frailty was narrowed to a range from 4.0% to 17.0%. The prevalence of the frailty status in our study population was 3.3%, slightly below the limit range found in the review, possibly due to the number of subjects enrolled in the present study (n=61). In a study encompassing "young old" subjects (aged 65 to 70; n=1283) the prevalence of frailty syndrome corresponded to 71.1% of robust, 26.4% of pre-frail and 2.5% of frail individuals (Danon-Hersch *et al.,* 2012). A low rate of frailty status was

observed, although this can be explained by the age of individuals included in the study ranging between 65-70 years (mean age 68.0±1.4), while in our study the age ranged between 65 and 95 years old (mean age of 77.3±7.1).

Several studies have shown the prevalence of pre-frail individuals higher than frail (Fried *et al*., 2001; Fernandez-Garrido *et al*., 2014). Results of our study corroborate with this finding (49.3% of pre-frailty vs 3.3% of frailty). In the systematic review of Collard and colleagues (2012), pre-frailty prevalence in studies using Fried's model was of 44.2% contrasting with 33.5% obtained in studies using broad frailty phenotype, as Rockwood Index Model (Collard *et al*., 2012).

Most studies reporting frailty prevalence in literature had their focus in Northern America population (Fried *et al.*, 2001; Puts *et al.*, 2005), which is a limitation since levels of frailty could be influenced by cultural, regional or political differences (Santos-Eggimann *et al.,* 2009). For this reason, Santos-Eggimann *et al.* (2009) carried out a cross-sectional analysis to evaluate the prevalence of frailty in 10 European countries within the SHARE project (Austria, Denmark, France, Germany, Greece, Italy, the Netherlands, Spain, Sweden and Switzerland) using for frailty assessment the Fried's Frailty Model. For the group ≥ 65 years the weighted proportion of frailty syndrome reported for 10-countries was 42.3% for pre-frailty and 17.0% for frailty. However, looking to each country individually the proportion of pre-frailty and frailty was higher in southern European countries compared to the northern. For France, Italy and Spain the weighted proportion found for pre-frailty status was respectively 43.6%, 45.6%, 50.9% while for frailty was 15.0%, 23.0% and 27.3%, respectively (Santos-Eggimann *et al*., 2009). In general, these data comply with our findings, and although the prevalence of frail individuals in our population was lower, mostly due to its limited size, is important to underline that among our pre-frail group, 40.0% manifested the presence of two components of frailty, thus near to frailty status (≥ three components).

Nevertheless, it should be noted that any effort to compare prevalence estimates across studies should be carefully conducted, since the definitions of the frailty criteria, the distribution of confounders (e.g., age and sex), and exclusion criteria differ between studies (Santos-Eggimann *et al*., 2009; Collard *et al*., 2012; Fernandez-Garrido *et al*., 2014).

Regarding the criteria that contribute to the classification of a frail phenotype, our results shown that the major contribution for pre-frail grouping was weakness (60.0%), followed by exhaustion (46.7%) and then, slowness and weight loss (both 16.7%). Furthermore, exhaustion, slowness and weakness were the criteria manifested by the subjects classified as frail (n=2) suggesting that pre-frail group manifested the same frailty criteria than the frail

group. In the study population, the most expressed positively criterion was weakness (62.5%), similarly with other studies (Xue *et al*., 2008; Danon-Hersch *et al.,* 2012;). Santos-Eggimann *et al*., (2009) also reported weakness as one of the most frequent criteria along with exhaustion. It was hypothesised that high scores of weakness might be linked with the onset of frailty, due to the loss of muscle quality and mass (sarcopenia) (Fernandez-Garrido *et al*., 2014).

As previously stated, the model used to assess frailty status influences the prevalence estimates of frailty groups (Ruiz *et al.,* 2012). Several epidemiological studies have corroborated the validity of Fried's Frailty Model to predict the risk of adverse outcomes in older adults, namely functional impairment, fracture, falls, hospitalization and death (Ensrud *et al*., 2008). The identification of frail individuals, prediction of outcomes and response of potential treatments are some requirements needed for an efficient frailty measurement and Fried's Model is one of the most currently suitable models based in these criteria (Dent *et al*., 2016).

Despite its popularity and widespread use, Fried's model is usually applied with few modifications on the original method proposed. More than 260 different versions can be found which limits the comparisons between studies using this model (Theou *et al*., 2015). A validated model with established methodology based on Frailty Fried Model is needed to be able to compare results and reach to potential conclusions.

Regarding the characteristics of the study population, both female and older individuals exhibited significant higher rates of frailty when compared with male and younger individuals.

Considering gender, in the frail group all individuals were women, in pre-frail group 76.7% were women and in robust group this percentage corresponds to 51.7%. This data is corroborated by several studies that reported higher rates of frailty (Fried *et al*., 2001; Morley *et al*., 2013) and pre-frailty (Fernandez-Garrido *et al*., 2014) in women. The higher prevalence of women than men in pre-frail and frail groups could be related with the higher number of women in older groups, considering their larger life expectancy (Collard *et al*., 2012). As stated herein, in Portugal in 2015, women had a life expectancy (at birth) of 83.3 years contrasting with 77.6 years for men (Pordata, 2015b). Since frailty is intimately linked with ageing, life expectancy of women could explain the higher rates of frailty observed (Collard *et al*., 2012).

The mean age of the study population is 77.3 (±7.1) years, being 74.8 (±1.1) for robust group, 79.2 (±1.3) for pre-frail group and 88.5 (±6.5) for frail group. These data are in accordance with previous studies that reported higher frailty rates regarding older elderly

ages (Collard *et al*., 2012). In the present study, a tendency for frailty increase with age can be observed. In fact, in robust and pre-frail groups (n=29 for robust group and n=30 for pre-frail group) a higher average mean age was obtained with increased frailty status. Age could influence the prevalence of frailty, nevertheless, it is known that chronological age *per se* could not justify the development of frailty syndrome due to the heterogeneity verified within groups with same age (Dent *et al*., 2016). Indeed, two decades ago some authors had already referred frailty as a broader concept for better assessing the heterogeneity of health status in elderly people, considering its high variety even in individuals with similar chronological age (Mitnitski *et al*., 2002).

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 27.9% reported being ex-smoker. Both robust and pre-frail groups were composed by a major number of never-smokers than by ex-smokers. No statistical significant difference was found between the prevalence of ex-smokers on robust group compared to pre-frail group. Both groups were also matched on the duration of smoking and in the period since stop smoking. Furthermore, although non-significant, in average pre-frail ex-smokers smoked for a longer period of time (24.6±6.8 vs 21.5±3.7) and stop smoking less years ago (29.3±7.5 vs 34.5±4.1) than robust ex-smokers, so pre-frail subjects were smokers during more years and left smoking more recently.

Further studies are needed to confirm if a link exists between frailty syndrome and second-hand smoke. Moreover, if second-hand smoke can potentially influence frailty it is probable that smoking can also influence it, thus more investigation should be carried out to confirm this effect. In general, our data shows the importance of including smoking habits as a variable when estimating frailty prevalence or investigating potentially factors promoting this syndrome onset.

Among elderly population, individuals respond differently to environmental agents due to their individual variability and susceptibility. 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). Environmental exposures have been linked with other age-related disorders, namely AD (Alzheimer's Disease) (Bakulski *et al.,* 2012; Yegambaram *et al.,* 2015). Since frailty is a multifactorial syndrome with no causal effects determined (Dent *et al*., 2016) environmental and

occupational exposures should be considered when scanning for risk factors related to this syndrome.

In this context, the relation of frailty syndrome with possible environmental and occupational exposures, current and past, was studied using a LTEQ (Lifetime Exposure Questionnaire) and a JEM (job-exposure matrix).

Environmental outdoor exposure-parameters evaluated included pollution sources located near home, such as, industrial production areas, farms, livestock farming, slaughterhouses, landfills, mines, and/or traffic zone. For indoor-air quality, the use of fireplace, pesticides (in plants and/or animals) and home ventilation habits were assessed. No particular exposure-parameter, current or past, outdoor or indoor, were significantly associated to either robust or pre-frail phenotype.

Very few studies have investigated the influence of environmental exposures on the frequency of frailty in older adults, the studies that did were focused on air pollution (Fougere *et al*. 2015). Myers *et al*. (2013) found an association between exposure to air particulate matter (i.e. $PM_{2.5}$, a known pollutant, associated with road traffic) with frailty status incidence in non-elderly population (≤65 years) after suffering a myocardial infarction. Although the study was not carried out in an older population, these findings might be taken in account for future research in elderly frail populations. Elderly is considered a susceptible subpopulation (Geller and Zenick, 2005) and frailty is characterized by a decrease in physiological reserve necessary to respond to exogenous stressors (Fielding, 2015). Furthermore, air particulate matter was one of the groups of chemicals of more risk for elderly according to EPA (Garcia-Esquinas *et al*., 2015a). Considering these evidences, if $PM_{2.5}$ was previous associated with frailty status after myocardial infarction in a non-elderly population, the link between air pollution and frailty syndrome in elderly groups should be investigated in future researches.

Using a JEM complemented with the information gathered by LTQE, the study population was classified according to the likelihood of occupational exposure to known hazardous compounds with bio-accumulating potential, namely pesticides, metals and organic solvents, but no significant association was found with the frequency of frail phenotypes.

Up-to-date, to our best knowledge, no studies were performed to investigate the association between occupational exposures and frailty syndrome neither studies relating pesticides and organic solvents exposures (occupational or not) with frailty syndrome. Previous studies have related the exposure to metals with age-related disorders, namely AD and PD, showing the role that these environmental pollutants may play in these diseases aetiology (Bakulski *et al*., 2012). A study from Garcia-Esquinas *et al*., (2015a) related the exposure

to two metals exposure, lead and cadmium with frailty syndrome. The results of that study indicated that blood lead concentrations revealed a positive dose-response with frailty, however for cadmium no relation with frailty was established (Garcia-Esquinas *et al.,* 2015a). In the present study, no relation between metals and frailty syndrome could be found: metals exposure was evaluated based in self-reporting and JEM data and no subgroups of metals were considered neither biological measures of blood concentration were performed. Furthermore, due to the limited population size, after categorizing individuals for frailty status and for the likelihood of occupational exposure to metals, small groups were originated, making difficult the association between those exposures and frailty syndrome prevalence. On the other hand, biological analysis could be performed to support the exposure self-reported (e.g. quantification of blood lead concentration could be an advantage to confirm if individuals exposed to lead display higher biological concentrations of this metal than non-exposed).

As stated herein, up-to-date few studies were carried out considering environmental and occupational exposures and frailty syndrome. Moreover, the scarce literature existent about this theme enhances the importance of the present study in this pioneer field.

Although frailty it is generally recognized to have a biological basis, no particular biological trait has been consistently associated to frailty status so far (Sanchez-Flores *et al.,* 2017).

Biomarkers of effect are biological indicators of the body's response to exposure and indicate early sub-clinical changes, which if sustained, may go on to have pathological consequences (Links *et al*., 1995). They either indicate early processes preceding disease or predict the development and presence of disease (by altered structure and/or function) (Kyrtopoulos, 2006). In terms of prevention, it is considered ideally if the biomarker is able to detect a biological alteration that is reversible. Genotoxic endpoint analyses are of great interest in risk assessment because they precede adverse health effects, thus offering a greater potential for preventive intervention (Mayeux, 2004).

It has been demonstrated, during the last two decades, the comet assay sensitivity to detect several degrees and type of DNA damage, thus providing useful information on primary effects induced by exposure to genotoxic substances (Collins, 2004) even in past exposures (Cavallo *et al*., 2014). Furthermore, oxidative stress is considered a risk factor for ageing (Coppedè and Migliore, 2009).

In the present study, no significant difference was found in the basal DNA damage and oxidative damage assessed by comet assay between robust group and pre-frail group. A paucity of studies have investigated the association between biomarkers and frailty prevalence, and very few used comet assay. In fact, most used different endpoints to

measure oxidative stress, genomic biomarkers and repair capacity, with both positive and negative outcomes. Nevertheless, our findings agreed with data available in the literature reporting no correlation between genomic instability and frailty (Sanchez-Flores *et al*., 2017). Results recently presented by Marcos-Pérez *et al.* (2017) shown no association between DNA damage, evaluated by comet assay, and frailty status in a cohort of 250 Spanish older adults (≥ 65 years). 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). In our study, no differences were found in the levels of DNA oxidative damage (oxidized purines) measured by comet assay between robust and pre-frail individuals, however it should be noted that the data found in the literature is related to frail individuals, a group that is not representative in our study population. For further conclusions a larger population with frail individuals are needed.

In human studies is important to assess the influence of major potential confounding factors such as gender, age and smoking habits in the biomarkers studied.

In the present study, gender and age were found to significantly influence the level of basal DNA damage and oxidative damage among the robust group.

Robust group women had a significant increase on basal DNA damage compared to robust men while robust individuals aged between 75 and 84 showed a significant increase on oxidative DNA damage compared with younger elderly. Gender, age and smoking habits were not confounders for the genotoxicity levels observed in pre-frail group. Marcos-Pérez et al. (2017) also found a non-significant increase of basal DNA damage on women compared to men in multivariate statistical analysis. In a major review carried out by Moller (2006) no effect of gender or age was found for basal DNA levels or oxidative levels (measured by FPG) respectively although a positive correlation was found for basal DNA damage with increasing age. Nevertheless, only three studies among 125 enrolled older adults (subjects aged 65 years or more), which may have influence the results. Indeed, age was found to effect other genotoxicity biomarkers (Bonassi *et al.*, 2001, 2011), this influence was associated with a progressive increase in spontaneous chromosome instability and the loss of efficiency in DNA repair mechanisms (Bolognesi *et al*., 1999).

The relation between some of the data collected in LTEQ (parameters with more than 20 individuals), on the levels of genotoxicity endpoints was analyzed. Significant differences were found regarding some exposure-related parameters, but only in the robust group.

Home-proximity to farming operation had a significant influence on the levels of basal DNA damage and oxidative DNA damage among the robust group, both endpoints were significantly decrease in these individuals compared to the ones not living near this activity. These findings are related to current exposure, no association was observed between previous local farming operation and the genotoxicity endpoints studies. Home-proximity to farming operation may imply that these subjects are living in a more rural area of the city, thus less exposed to a more contaminated urban environment.

In results recently presented by Marcos-Pérez *et al.* (2017), no significant statistical differences were observed on the repair capacity associated to frailty phenotypes, however a decrease was observed between robust, pre-frail and frail individuals (robust > pre-frail > frail). Future research should explore the link between repair capacity and frailty groups, taking in account the environmental exposures.

Among robust individuals those consuming home-produced vegetables had a significant decrease on the level of both basal DNA damage and oxidative DNA damage compared to those not consuming these vegetables. Moreover, those who consumed more vegetables home-produced were classified as robust. A study of Garcia-Esquinas *et al.,* (2016) referred that the consumption of fruit and vegetables may be a protective factor against frailty. In that study, 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 the present study, the source of the vegetables consumed were asked, but the frequency was not, so this variable was not analysed. Nonetheless is safer to speculate that subjects producing or having assess to home-produced vegetables may consume vegetables more frequently than those who have no easy access and probably have to buy it.

The influence of fruit/vegetables intake on basal DNA damage and oxidative DNA damage evaluated by the comet assay has been largely described with both positive and negative outcomes. (Brevik *et al*., 2011). In Brevik *et al.* (2011) study although no significant variation in oxidative DNA levels was observed the repair capacity (BER) was increased with the increase of fruits and vegetables intake, of note, the study was a dietary intervention lasting 8 weeks. Epidemiological studies consistently report that a balanced diet rich in fruit and vegetables is associated with a reduced risk of cancer and heart disease (Duthie *et al.*, 2006). Indeed, fruit and vegetables are rich in several phytochemicals and antioxidants that

inactivate reactive oxygen species (ROS) involved in the initiation or progression of chronic diseases.

Previous studies linked the anti-oxidative vitamins and phytochemicals of vegetables and fruits with prevention of oxidative stress-related diseases prevention however inter-individual differences in body antioxidant capacity and in the risk of diseases after antioxidants supplements consumption was reported (Yuan *et al*., 2011).

Up-to-date no studies were performed in a frailty population, linking the level of DNA damage with the consumption of fruits. Due to vulnerability of frailty individuals, studies conducted in this population could be distinct of previous studies in other target population. Thus, further studies should improve the knowledge about the effect of consumption of home-produced vegetables and the decrease in DNA damage and, consequently their potential to prevent frailty. The findings in the present study also support the inclusion of other exposure-related parameters assessed in LTEQ, such as the frequency of fruit and vegetables consumption. It also emphasizes diet as one possible influencing factor to be included in human biomonitoring studies investigating frailty syndrome.

The results of the present study confirm the use of comet assay technique as a biomarker of recent damage, since all the associations observed in this study between DNA damage (basal and oxidative) and exposure were related to current exposures (home-produced vegetables consumption and living near of farming operation activities). The inclusion of a complementary biomarker of effect for measuring chronic exposures, namely micronucleus assay (for lymphocytes and buccal cells) (Sinitsky & Druzhinin 2014) might be of interest in future researches for deepen the knowledge about link between previous exposures and DNA damage. Micronucleus assay is a widely used method in human biomonitoring studies (Holland et al 2008), although is a time-consuming method (compared to comet assay technique) and for this reason its inclusion in the present study were unable. On the other hand, the associations observed between the exposures assessed with Lifetime Exposure Questionnaire and the endpoints studies, confirms the importance of using tools as questionnaires in human biomonitoring studies.

CONCLUSION

Frailty syndrome is an age-related disorder with high prevalence in elderly populations. The ageing worldwide phenomenon boosted some researchers to study what causes frailty syndrome. Few studies linked environmental exposures with the frailty syndrome until now. Although some of these studies suggested a relation between environmental pollutants and frailty syndrome, no conclusive findings were reported.

In the present study, several factors such as environmental and occupational exposures aimed to be related with frailty syndrome. A significant relation between frailty and women and between frailty and older age groups was observed. Second-hand smoking also shown to be related with frailty status. No significant differences were found between robust and pre-frail groups on levels of genotoxicity parameters, evaluated by comet assay. Association between frailty status and home-produced vegetables consumption was found, with robust individuals consuming more vegetables from this source compared to pre-frail individuals. Furthermore, associations between the intake of these vegetables and DNA damage in robust groups was found, robust individuals including these aliments in their diet showed significant lower DNA damage than robust individuals. Gender and age were shown to be potential confounders factors for comet assay endpoints within the robust group. Lastly, also within the robust group, a relation was observed related to household-proximity to farming operation.

The strengths of this study encompass the complete methodology of study, that englobe the use of a validated measure for frailty syndrome assessment (Fried's frailty model), a self-report Lifetime Exposure Questionnaire about current and previous critical exposures, a Job Exposure Matrix for occupational exposure assessment, and, lastly, a quantification of basal and oxidative damage using comet assay method, commonly used in human biomonitoring studies. Furthermore, regarding frailty phenotype assessment, the use of accurate and valid instruments of measurement, such as the flat mechanic scale (SECA® 761), the stadiometer (SECA® 213) and the dynamometer (*JAMAR*® 5030J1), allowed robust data on these parameters.

The limitations of this study include the reduced population size, making difficult the comparisons between frailty status groups. Furthermore, due to the size of frail group, no statistical analysis between this group and the robust and pre-frail groups were performed.

Future studies should include a larger population, with representative number in each frailty group (robust, pre-frail and frail). In addition, taking in account the recent evidence on the association of environmental contaminants with age-related diseases, risk factors concerning environmental and occupational exposures should be further investigated as potentially influence frailty syndrome. Other biomarkers, effect or exposure, more sensible

to chronic exposures should also be included. The identification of people at risk of frailty will allow implementing preventive actions and specializing geriatric care, improving the quality of life in old age and reducing healthcare costs.

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