A CALCIUM-CENTERED SOCIO-ECOLOGICAL MODEL OF PROSTATE CANCER DISPARITIES: PRELIMINARY STUDIES AND FINDINGS

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Abstract

Western studies have established that men from African descent are disproportionally affected by prostate cancer (PCa). Annual incidence rates in this population vary from 1.5 to 2 times when compared to their counterparts from other racial groups. They also record the worse outcomes in terms of prognosis. Additionally, with the rise of PCa in Subsaharan Africa, new cancer control policies and programs are increasingly demanded. Understanding therefore, factors that underpin racial inequality in distribution and especially why the disease preferentially niches in African males can help better address PCa in both Western and Subsaharan countries. There is also the potential to develop new therapeutic options. A genetic susceptibility was first hypothesized, however available data suggest that they only account for less than 20% of the cases. Current findings from epidemiological and molecular investigations suggest an important role of complex and dynamic environmental interactions involving the different levels of calcium regulation. Using a multi-method design, this research aims at developing an integrative mechanistic model of PCa. We argue that given the versatile and ubiquitous role calcium plays in nutrition, physical environment, and in key cellular processes, that mineral cation is central to prostate tumorigenesis and in shaping its populational distribution. Thus a tree-level investigation was conducted: (*i*) a critical analysis and synthesis of empirical evidence on calcium interactions with cancer mechanisms (*ii*) a population-wide prospective cohort study of calcium intake patterns in a group of Subsaharan males in Côte d'Ivoire, namely the African Prostate Cancer Study (APCS) (*iii*) a proteomics research investigating the responses of prostate cancer cell lines when exposed to a high affinity synthetic calcium binding peptide. This monograph describes the research methods, instruments design and validation and the preliminary findings of the ongoing research, portions of which have already been published, presented at two international cancer seminars or under review. Findings at this stage include: mechanistic models of prostate cancer differential distribution and outcomes, a novel calcium questionnaire specific to African diet, synthesis of a high affinity calcium-binding peptide (Peptide#1). New concepts and constructs related to prostatic carcinogenesis have been developed as well.

Keywords : **Calcium - Prostate cancer - racial inequality - socio-ecological model - transgenerational epigenetic inheritance.**

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LIST OF ABBREVIATIONS

APCS - African Prostate Cancer Study

Ca2+ - Calcium

CASR- Calcium Sensing Receptors

CBP - Calcium Binding Peptide

DRE - Digital Rectal Examination

EDC - Endocrine Disrupting Chemicals

FFQ - Food Frequency Questionnaire

PCa - Prostate cancer

PSA-Prostate Specific Antigen

R - Recall

STIM/Orai - Stromal Interaction Molecules

SOCE - Store Operated Channels

SES - Socio-economic Status

TEI- Transgenerational epigenetic inheritance

VDRE - Vitamin D Receptors Elements

Chapter 1 : Introduction

1. Statement of Problem

Prostate Cancer (PCa) is a malignant tumor developed in the human prostate gland[1]. It's the most common cancer in men. It's also a leading cause of death by malignancy, only second to melanomas[2]. In 2018, over 1.3 million cases were diagnosed worldwide, representing 7% of all cancer sites, with 360 000 deaths [3]. Compared to 2008 numbers, it was a surplus of 200 000 new cases and as much new deaths[4]. Global cancer data suggest that the decadal increase in both incidence and mortality is mostly attributable to the rise of the disease in the Developing Countries [5]. In effect, estimates from the 2014 World Cancer Report asserted that cancer mortality burden has now shifted to the South. It's been even forecasted that by 2030, cancer and mostly prostatic malignancies will be the major cause of morbi-mortality in emerging Nations [5]. Along with these high numbers, prostate cancer also displays a singular distribution: men from African descent are disproportionately affected when compared to their counterparts from other races/ethnicities. In the US for example, African American males (AA) account for 2/3 of the national burden in terms of incidence and fatal outcomes[6]. The same trend is described along the routes of the African Diaspora: in 1998 a Jamaican study reported the highest incidence in the world (308/100 000) associated with the worst outcomes[7]. Tackling prostate cancer in Subsaharan Africa, where similar concerns are rising, will most likely be amongst the major public and population health matters of interest in the coming decades[8]. However, without a clear understanding of the determinants and factors that shape its distribution patterns, a sustainable and affordable prevention program for developing countries is unenvisageable. Current *prevention* methods based on wide-spread Prostate Specific Antigen (PSA) screening programs are costly and not necessarily efficient especially for less affluent nations [9]. Genetic factors have been suggested to explain the relatively increased susceptibly of African males [10], but it's now established that they only account for less than 20% of the cases[11]. In the recent years, observational studies have emphasized the influential role of multiple environmental factors, nutrition in particular [12, 13]. Diets rich in dairy products and calcium from animal sources have been intensely debated over the last two decades [14]. However, findings have been inconsistent and it remains unclear, to date from an epidemiological standpoint what's the precise role of calcium and what biological pathways might be involved. On the other hand though, studies at the cellular and molecular levels emphasize the critical role calcium might play in prostate tumorigenesis[15, 16]. Multiple and complex mechanisms have been

suggested, although very few attempts have been made to provide a comprehensive model of the tumor, that would bridge, at least at the conceptual level, knowledge from various field including social, geographic and biological. The purpose therefore of this research is to examine, from a multi-disciplinary perspective a calcium theory of prostate cancer racial disparities and suggest a coherent and integrative vulnerability model to the disease. By « *Coherent »* it should be understood that the research will evidence a causal relationship between different concepts in the prostate cancer phenomenology and *« integrative* » here signifies that the contemplated framework will aim at reconciling epidemiological findings, social knowledge and theories and biological pathways, through including, along with diet, multiple risk factors pertaining to all three levels of the socio-ecological scale. The main argument in this research is that calcium metabolism, in its various connections with diet and other environmental exposures might be a pathway of prime importance whereby prostate cancer *gets under the skin* and displays a racially differenciated distribution. Capturing such connections through a logical pathway, would help design new community-based prevention programs to better complement the biomedical paradigm. The approach also has the potential to unlash new calcium-based therapeutical options.

The investigation is undertaken through a doctoral and post-doctoral research and the present monograph describes the study rationale, design and methods. We also reports on the preliminary findings of the portion of the research that was conducted between 2016 and 2019, and already published or under review. The questions guiding this research are described below.

2. Research Questions

The main question guiding our investigation can be framed as followed : *How are broad environmental and social factors influencing internal body calcium, such as dairy-rich diets, or other calcium metabolism influencers, connected to the series of biological events that lead to prostatic tumorigenesis and, in this molecular phenomenology, why are African males more susceptible than males from other ethnicities ?*

Given the complexity of this question and its multi-disciplinary nature, it’s more appropriate and operational that it be delineated in 3 investigational queries:

1. To what extent the existing body of empirical evidence supports the role of calcium in cancer ? What causal mechanisms could be suggested linking prostate tumorigenesis to nutritional calcium and to other environmental calcium metabolism influencers?

2. Is dietary calcium associated with the increase in incidence of prostate cancer in Subsaharan Africa men? What are intake patterns in this population and how does it compare with Western Africans ?

3. Is there a differentiated cellular response when prostate cancer cell lines isolated from patients with different ethno-racial backgrounds are exposed to a synthetic calcium binding peptide ? What protein(s) or group of protein are then deregulated ?

3. Research Objectives

With these questions come a series of different objectives. Thus this research will aim :

1. To critically synthesize and analyze the body of empirical evidence linking molecular calcium to prostate tumor initiation, promotion and progression;

2. To identify the principal molecular mechanisms involved in a calcium-centered hypothesis of carcinogenesis;

3. To suggest a new theoretical model of cancer causation based on molecular calcium;

4. To develop a standardized and culturally adapted instrument for measuring nutritional calcium in an African diet type and describe nutritional calcium intake patterns in a population of Subsaharan African males;

5. To test the correlation between nutritional calcium intake and incident prostate cancer rates in a Subsaharan African male population;

6. To compare nutritional calcium intake and prostate cancer risk between two African male populations, with a common genetic ancestry, but diverging by nutritional calcium intake patterns and other environmental exposures;

7. To synthesize a highly calcium specific peptide that would influence calcium metabolism in prostate cancer cell lines;

8. To evaluate and compare the responses of prostate cancer cell lines of African origin to cell lines of Caucasian origin when both are exposed to a high affinity calcium binding peptide

9. To identify the deregulated protein (s) or group of proteins when two different prostate cancer cell lines one from African origin and another from Caucasian origin are experimentally exposed to a high affinity synthetic calcium binding peptide

4. Study Context and Justification

The research was conducted at the University of Ottawa, Canada (uOttawa), within the Interdisciplinary School of Health Science and then at the Laboratory of Chronic Diseases both affiliated respectively with the Faculty of Health Sciences and with the Faculty of Medicine. Another portion of our investigation, the field research, was conducted in Bouaké, central Côte d'Ivoire, West Africa at the Service d'Urologie of the University of Bouaké, Université Alassane Ouattara (UAO). Given the increase in prostate cancer incidence in Côte d'Ivoire in the recent years, the Service d'Urologie of the Bouaké University started a community-based prevention program in conjunction with different associations of retired people back in 2014. The aim of the project was a long term prospective follow up of a cohort of retired males with annual check ups based on PSA testing and digital rectal examination (DRE). The follow up also included a health education program to increase awareness of prostate cancer, physical activity and diet in the elderlies. Another aspect involved studying the barriers and facilitators of a large scale prostate cancer early detection program in the country. Our research benefited from the context and infrastructures already established by that ongoing study and eased access to the targeted populations. Similarly, the laboratory study of our investigation benefited from the expertise of the researchers of the uOttawa School of Health Sciences and Faculty of Medicine. The Chronic Disease research program was developed to promote innovative therapeutic options targeting new

molecular pathways for diseases such as obesity, cancer or diabetes. Also, the University of Ottawa, through the late Institute of Population Health and now through the School of Health Sciences was a leading institution in the thematic of social determinants of health and health promotion programs over the last decades. The complex problematic of prostate cancer affecting and afflicting mostly men from African ancestry has been a subject of interest for both the School and the Institute in their aim to address health disparities at the global level. Our research integrates the broader debate of the relationship between genes and environment, nature and nurture applied to cancer. Genetics is certainly one of the most promising future of the biomedical paradigm. New possibilities have emerged connecting different forms or expressions of disease to a set of genes, paving the way to what will be known as *Precision Medecine*. But the limitations of the biomedical model of disease in general and of cancer in particular are best seen when we compare populations with similar genetic ancestry yet exposed to different environment. In our study multiple comparisons are conducted: A *comparison between* two populations of African males: the Subsaharan and the Western, in reference to those who have been established in the Western hemisphere for generations. Of these, the African American males are the most investigated. The two African populations share a common genetic ancestry but radically differ by history, diet, place, social structures factors well established to affect population health. In age-adjusted studies the Subsaharans record a relatively low incidence rate compared to the Americans, although now the gap is narrowing, given the steady increase of prostate cancer incidence rates in Subsaharan Africa. But our research also conducts a *comparison within* the Subsaharan population itself, but at two different points in time. The retired population in Africa today still holds on to the traditional African diet and lifestyle with very limited change, whereas the new generations have embraced a more westernized way of life. It's of population health interest to appraise how change in diet, especially in calcium intake patterns, has affected prostate cancer incidence. Finally, we also conduct a *comparison inter* : the proteomics portion compares the way prostate cell lines from different races/ethnicities respond to the same calcium-related stimulus. The idea here is to confirm a specific action of calcium but also to locate the level where the difference is made either environmentally or genetically.

Chapter 2: Literature Review

1. Prostate Cancer Disparity

1.1. Cancer of the Prostate Gland

The prostate is a compound tubuloalveolar exocrine organ belonging to an anatomical system of three (3) specialized hormonal tissues commonly referred to as the male accessory glands. The organ derives from the embryonic mesenchyme of the urogenital sinus, under the stimulation of fetal testosterone[17]. The prostate gland increases its weight slowly from 2g in childhood to 20g at puberty. However, toward the end of the third decade of life the gland starts growing slowly marking the onset of benign prostatic hypertrophy. From a functional standpoint, the prostate produces the essential of the ejaculate consisting mainly in proteins involved in semen gelation, coagulation and liquefaction. The most known secretion is the Prostate Specific Antigen (PSA), a kallikrein-type serine protease that dissolves the coagulum. Under normal conditions, PSA is not, or at least minimally, found in the blood stream[18]. However, when the normal architecture of the gland is disrupted as seen in chronic inflammation and in cancer, PSA can be detected in the blood. To date, along with digital rectal examination (DRE) and ultrasound, PSA is the most utilized diagnostic lab. Exam for prostate cancers, which are dominated mostly by prostatic adenocarcinomas. In effect, adenocarcinomas represent over 95% of the histologic types of all PCas[19]. The tumor evolves through a multistep stage, from a variety of precancerous lesions, referred to as prostatic intraepithelial neoplasms (PIN)[20]. The clinical presentation is one of a patient generally in his fifty's consulting for some urinary symptoms. More frequently, especially in setting with no systematic checking, the disease is discovered at a more advanced stage. Metastasis and secondary location mark the gravity of prostatic adenocarcinoma, and the most frequent secondary localization is the bony tissue.

1.2. Incidence and Mortality

As mentioned earlier, PCa is the second leading cause of mortality by malignancy after melanomas[21]. Although, along with other cancer types, the recent decades have witnessed a decline in mortality in Western countries. It has been estimated that approximately ~ 2% decline in mortality was recorded every year since 1990[22]. Factors accounting for the decrease are

uncertain. Most researchers argue that it could result from the combined effects of widespread PSA screening, testing, increased awareness and better clinical management protocols. Nonetheless, it should be noted that that positive trend is grieved with structural disparities, both within and between countries. For example, US data from the SEER Program (Surveillance Epidemiology and End Results) elicit a gradient in outcome attributed to both socio-economic status and ethnicity [23]. Socio-economic status, approximated by income and education, has been established to be the main factor accounting for health disparities, as emphasized decades ago by the WHO Commission on Social Determinants[24]. This happens to be verified for diseases in general, but the relationship is even stronger for chronic maladies such as prostate cancer[25]. However, what is alarming for PCa, is the increase in incidence in the Global South. Indeed as mentioned earlier, since 2012, the mortality burden of PCa has shifted to developing countries. Data suggest that they represent represent about 52% of the worldwide mortality burden[5]. Both mortality and incidence in these countries are on a steady increase[3]. The change has been paralleled to increasing urbanization and westernization of lifestyles [8]. Chu et al. reported that the more local habits such as diet, lifestyle, behavior, and living conditions changed, the more prostate cancer cases were reported. The literature reports different explanatory factors: firstly, an inflationist effect of widespread access to modern healthcare and PSA testing, similar to what was seen earlier in the 1990s, in Western countries. Secondly, the relative increase in life expectancy : Southern Nations have recorded a substantive increase in life expectancy over the recent decades, and therefore diseases that normally present in the fifth decade of life are now becoming more common[8]. Thirdly, the increase in awareness in the general population might have *revealed* a disease that was already highly prevalent in the African population but was ignored for various reasons [26]. This later argument of a *statistical artifact* is nonetheless more debatable. Comparative studies, after adjustment for age, access to healthcare, income, education levels, or other factors, still outline a persistent gradient in incidence in the emerging nations, especially in Africa [26]. Countries like Nigeria and Côte d'Ivoire in the West coast of Africa rank among the most affected and Zimbabwe, Kenya and Tanzania are the most affected on the Eastern coast with incidence annual rates varying from 11 to 37 /100 000 which numbers are still lower when compared to African Americans or Diaspora Africans [27].

1.3. Risk factors

Prostate cancer is probably among the most investigated of all cancers. A quick search on Pubmed with restrictions to articles published over the last 5 years was able to retrieve almost 46 000 articles; in comparison colon cancer showed only 28 000 articles. And one of the reason of that constantly renewed interest lies in the multiplicity of identified risk factors. There is also the impression in the cancer research community that a treatment and a prevention is at hand. Four different groups of risk factors can be identified, with certainly an interconnection and correlation between them : Demographic, hormonal, behavioral/lifestyle and genetic [28]. It's probably that multiplicity that paradoxically creates the optimism in the prostate cancer research community with the hope to develop a primary prevention model of the disease. Such a model, as we argue in this research, might be possible at the condition that we understand and develop a mechanistic framework that logically integrates all these risks factors.

1.3.1. Demographic factors and anthropometric measures

They mostly include include increased Body Mass Index (BMI), age, and race, recent studies suggest that height must play a role as well. BMI will be discussed in another paragraph. It's estimated that 63% of incident prostate cancer happens in men aged 65 or older[29]. Available studies suggest that the age line is lower in males from African ancestry, as they appear to be affected at a younger age generally around 60 if not earlier [30]. But the general consensus is that prostatic carcinomas appear after the fifth decade of life. The racial divide of the disease is probably the most stunning feature of prostate cancer epidemiology. Incidence vary along the lines of the different male populations, and Asian males for example account for the less vulnerable population of all males. It should also be noted that this is more verified when the male populations are evaluated in their original habitat, suggesting the crucial role of social, environmental, and cultural factors including, but certainly not limited to, diet. On the other end of the spectrum, African males records a 1.5 higher incidence than their Caucasians counterparts and 2 times higher than Asian males. Evidence of this surfaced in the early 1990s, through retrospective analyses conducted on databases from the SEER [31]. Today there is a consensus from all investigations, that along with family history and *age, race* is the most well -grounded risk factor of prostatic tumor[30].

1.3.2. Hormonal factors

Growth factors (GF) and hormones, mostly androgenic hormones such as testosterone, have been known for a long time to be an important biological factor in prostatic tumorigenesis [32]. A posteriori evidence of this is provided by the various anti-androgen therapies that serve today as a reference treatment for the disease. In fact, the central role played by the hormones here derive from both embryologic and histologic factors inherent to the prostate gland as quickly reviewed at the beginning of this chapter. The effects of testosterone on the prostatic glandular tissue from embryonic life to older age would quasi determine the fate of the gland as we have mentioned[17]. Continuous hormonal stimulation lead to the increase in volume of the gland and is a main factor in cancerous transformation. Tumors displaying the androgen receptors (AR) are of a better prognosis when compared to tumors that don't, and as mentioned earlier, hormonotherapy is the first line of treatment and resistance or evasion to hormone treatment is always perceived as a darkening of the prognosis. It should be noted that it this strong role, played by male hormones that *connect* the physiopathology of prostate tumor development to the broader environment. In effects, substances known as endocrine disrupting chemicals (EDC) or endocrine disruptors (EC) [33] are known to play a role in prostate tumorigenesis . That mechanism has been suspected in professions exposing directly workers to the EDCs such as workers in chemical industry or even in farmers [34]. Which will account certainly in the disproportionate distribution of the disease. Epithelial Growth factors (EGF) have been identified as contributing to metastasis[35]. Chronic inflammation could also be mentioned in this category although not directly related to hormones, the molecular mechanisms being quite similar[20].

1.3.3. Behavior, lifestyle and diet

As a general rule, physical activity has been inversely correlated to cancer risk and Prostate cancer is not an exception to that rule as attested by The Continuous Update Project (CUP) reports [36, 37]. The protective effect of physical activity has been attributed to the increase in energy catabolism, and a decrease in the accumulation of end products of the metabolism such as the Advanced Glycation End Products (AGE) and their Receptors (RAGE) [38]. Physical activity belongs to a broader interacting loop in which major leverage points include *diet* and *obesity*. The role of diet and nutrition has been abundantly discussed in the literature. Starting around 2007, an increased research was conducted to identify food association with prostate cancer perceived by some author as a possible nutritional disease [13]. Indeed, that intense research work has led to the evidencing of some protective effects of food or their derivative such as *lycopene* in tomato

products [39], quercetine[40] or an increase in fresh fruit and vegetable consumption. On the other end of the spectrum however, other food and cooking practices have been suggested as detrimental to the prostate gland[41]. Increase red meat consumption, and processed meat and animal food in general has been positively correlated to an augmentation in annual incidence rates [42]. High temperature cooking methods such as grilling and barbecuing through the production of heterocyclic amines (HCAs) and polycyclic aromatic hydrocarbons (PAHs) which are well established carcinogens are suspected to be part the biological mediators of cooking practices in prostate tumor development [43]. There has been, however, a long standing debate on the role of dairy products consumption. Earlier studies to suggest the detrimental role of dairy surfaced at the beginning of the century with research conducted in North America [44]. Milk being a central element in Western diet, it didn't take long for various studies to be conducted around the world using different methodologies : prospective cohort studies, cross-sectional studies, case-control studies and their respective meta-analyses. Very important studies were conducted during that time for example : SUIVIMAX in Europe[45], the Physician Health Study (PHS) in the US[46], the multiethnic cohort in (MEC) Hawaï[47] , the Finnish Study[48]. By 2007, an important number of publications has been made to the point where it became necessary for the scientific community to have a panel of experts to review the question. It was the Continuous Update Project of Prostate Cancer risk in relation to nutrition, physical activity and obesity, supported by the World Cancer Funds among others. The first report of the CUP was published back in 2007 and established that the evidence relating prostate cancer risk to dairy consumption was *strong*[49]. In other words, a meta-analysis of studies published during this period, there was a positive and credible correlation between dairy consumption a proxy of calcium intake, and prostate cancer risk. And calcium in dairy was the main contributing factor as suggested by Mitrou[48]. A good summary of this period is provided by Li-Qiang and coworkers in a seminal work published in 2007[50]. Starting in 2008 a series of new publications criticized the association. Bristow and colleague provide a good summary of this works [51]. Non Western studies however, have provided support to the association [52] and as recent as in 2015 and 2017 new studies have surfaced providing additional support [53, 54]. However, a new report released by the CUP in 2014 and then in 2018, downgraded the strength of the evidence from *strong* to *weak*[36]. It's interesting to note that although African ancestry is an established causal factor, as we mentioned earlier, very few studies in the debate have been directed to African American populations and none was conducted in Africa. Findings in African American males in studies where they were involved did not show much differences with their caucasians counterparts, although Rowland and coworkers found a dual effect

in their research : a positive association with calcium intake at certain intake levels and a protective effect at low intake levels [55]. Research focusing on the most at-risk population is lacking in the debate and if such is conducted it is expected to shed new insights for a better understanding of calcium role. This account for one of the reasons among others to include in our study a specific study on the Subsaharan males.

1.3.4. Genetics

When the disproportionate burden of prostate cancer in African American males was evidenced by SEER studies over a decade ago[6], genetic factors were the first to be suspected[10, 56]. Genome-Wide Associations Studies (GWAS) are research aimed at stratifying the general population based on allele frequency differences between cases and controls in relation to systematic ancestry differences. The method has been popularized by Price and coworkers at the beginning of the 21st century [57]. GWAS have identified over forty (40) single nucleotide polymorphisms (SNPs) , specific regions, also referred to as *loci*, of the chromosome showing either a *loss* or a *gain*, that are associated to increased susceptibility to prostate cancer[10, 58]. The most studied SNPs to date are 6q13-22, 8p21, 13q13-14, and 16q11-24 and gains of 7p21 and 8q24 [59]. Fueling the genetic argument is also the fact that for all race/ethnic groups, a family history of prostate cancer has been correlated to an increased risk [5]. In a US based study, Hayes and colleagues established that both African and European American men had over three times the odds of the disease if they have a family history of prostate cancer when compared to men with no first degree relative affected[60]. However, in a small study comprised only of African American men, Bebee-Dimmer and coworkers reported that having a sister with a history of breast cancer was also positively associated with prostate cancer occurrence [61]. There is also a racial difference in the profile of the disease. The prostatic tumor in men from an African ancestry, appear at a relatively younger age as mentioned above, but also is associated with rapid progression and the worse outcomes.

2. Investigating prostate cancer racial differences

How can such a variety of risk factors explain prostate cancer disproportionate burden on males from an African descent especially those in the African Diaspora? How can they explain the rapidly increasing incidence rates in Subsaharan African? It is clear that more understanding of prostate cancer vulnerability mechanisms is needed as it could also potential open the door to other cancers as well. Two major disciplines can be distinguished in investigating the racial differences in the

disease: biological studies and observational studies. The biological approach includes investigations focussing on hormones, inflammation or on genes whereas epidemiological studies focus on geography, demographics, behavior and most importantly diet. The question of nutrition and cancer has gained strong momentum as illustrated by the Harvard-led *Pooling Project of Prospective studies on Nutrition and Cancer*[62]. Notorious cohort studies are included in the project some we have mentioned earlier, such as the Multi-Ethnic Cohort (MEC) study in Hawaï[47], the NIH -led American Association of Retired Persons (AARP) study [28], the well-known Physician Health Study (PSH) to name a few. As stated earlier, no African study or study focusing exclusively on males from African descent are included in the Project although African ancestry is among the most established risk factors. The broad spectrum of research on prostate cancer causation emphasizes and legitimates the need for a unifying model that would connect all the disciplines. Four major theories can be traced in the literature.

2.1. Schwartz-Rowland Vitamin D and Calcium theory

The *Calcium-Vitamin D hypothesis* of prostate cancer disparities first emerged in the early 90s under Schwartz works [63]. Proponents of this theory hold the view that ethnic and geographic differences in risk distribution are rather attributable to differential exposure to sunlight, hence to Vitamin D metabolism. The Vitamin D component has even been evidenced in other cancer types in countries with high seasonal sunlight variations[64]. The theory was further refined when empirical studies showed the impact of molecular calcium on regulation of Vitamin D Receptors Elements (VDRE) [65]and even directly on prostate cancer cells[66]. In 2012, Rowland and coworkers investigated the potential role of calcium absorption through a multidisciplinary research combining nutrition and genetics. The authors reported that study participants with the Vitamin D Receptor Elements (VDRE) CDX2 GG genotype were low absorbers of nutritional calcium and were associated with relatively lower risk of prostate cancer[67]. This effect was reported as a *calcium gene interaction*. The limitation of the *Calcium-Vitamin D theory* however was evidence that came from observational studies. Africans living in the Caribbean islands : Jamaica, Guyana, Brazil, account among the highest prostate cancer incidence in the world while by geographic position, they should have adequate exposure to sunlight. The model therefore is more likely to explain *interethnic vulnerability* which is the differential incidence among males from the same ethnic background but not *interethnic vulnerability.*

2.2.The genetic polymorphism theory

As mentioned earlier, specific chromatin regions have been associated with increased risk. However , available studies suggest that less than 20% of the prostate cancer patients have been identified with the vulnerability nucleotides[11]. Also studies in identical twins result in diverging conclusions, and most importantly age-adjusted studies clearly support that males in Subsaharan Africa has a lesser vulnerability when compared to their Diaspora counterparts at least before the Westernization movement on the Continent[8]. But the fact that there is an established familial history of prostate cancer strongly support a some of *inheritable factor* playing a role in the disease.

2.3. The environmental theory

The environmental theory extends to all factors outside the body but not focussing on a specific *vector,* whereas Schwartz-Rowland's theory focusses on Vitamin D and Calcium. This then includes a variety of risk factors such as nutrition and diet, occupational and non occupational exposures to hormonal disruptors and chronic inflammation, healthy behavior. The panel of experts of Continuous Update Project (CUP) on prostate cancer risk aims at evaluating the strength of the evidence linking those factors to prostate cancer risk. Their findings have been regularly published : in 2007 for the first report, 2014 for the second and 2018 for the most recent[36]. Giovannuchi has been a strong proponent of that theory especially for diet. His work on tomato products and on dairy product have a certain authority to date[39]. However, the environmental theory falls short to explain, on its own, prostate cancer disparities. Nutritional studies have not shown a significant difference in dietary intake patterns between African American males and European American Males [46, 56]although some have suggested differences in cooking patterns[68].

2.4. Socio-economic and cultural theory

The socio-economic theory is not very popular in the prostate cancer research community. It mainly derives from observation that there is a difference in prostate cancer outcomes *before* prostatectomy but after surgery relapse rates and outcomes are the same across the different racial, income and education levels. In that approach, the racial gradient is not but a reflection of a socio-economic gradient[23]. If the theory could largely account for racial differences in outcomes as suggested by Ravery et al. [69] it doesn't explain differences in incidence. The question remains how the socio-economic disparities affecting Africans Americans *get under the skin?* And are Asians Americans

better off than European Americans ? Because the former report a relatively low incidence rate of prostate cancer when compared to the latter. Therefore the socio-economic theory like the former theories fall short too in explaining the peculiar distribution of the disease.

2.5. Syncretic theories

An attempt to combine different explanatory theories of prostate cancer disparities has been made through the concept of *gene-environment interaction* whereby environmental factors act synergistically with genetic predispositions to induce carcinogenesis[70]. A similar hypothesis was recently formulated with breast cancer [71]. One of the limitations of the approach is the lack of *connector* or *mediator* that would connect environmental factors and their genetic counterparts in such a way that cancer disparities could be satisfactorily explained. The limitation of this otherwise interesting model, is that, it championed a preexisting genetic polymorphism that is not supported at the race level. In clear, no study to date has evidenced cellular traits that would explain vulnerability at the phenotypic level. Furthermore, the model fails to explain why highest incidence rates in the world have been reported in African men specifically from the Caribbeans: Jamaica- Guyana-Brazil. Also, how do we explain the inconsistencies of epidemiological studies on the role of calcium ? Not only there is a pressing need for a unifying model, but such a model should also be comprehensive to integrate the best explanatory theory possible for the quasi totality, if possible, of the risk factors. Socio-ecological models, through the population health paradigm appears to be the best option available so far.

Chapter 3 : Research Theoretical Framework and General Design

1. The Population Health Approach

As stated by Rose, the science of population health distinguishes between the causes of individual cases and the causes of patterns of incidence in the population[72]. The baseline assumption in the field is that the entire risk for the general population is decreased by an appropriate focus on the most at-risk population. Also, whenever entire populational subgroups share a disproportionate burden of a common disease, the chain of causation of such illness should be expanded to explore the factors that support such a particular distribution. In general, these factors are deeply nested in the ecosystem, and are best referred to as the *determinants*. The epistemological quest in Population Health as a science, is therefore to define the contours of such influences using a modeling approach. Such models are called *ecological,* to use an environmental metaphor*,* and were first imported in the realm of public health in the mid 60s emerging from two main sources: the Lewin's *field theory* (1968), recommending that *all* factors located in a "field" be considered to explain behavior, and Kelly's *community psychology* (1968) in which the term "*ecology*" is used to describe the dynamic interdependency between structures and processes within an ecosystem. McLaren & Hawe summarize the ecological theory of health as a comprehensive approach, in which disease is perceived as the resultant of a complex interaction unfolding at multiple levels of influences: historical, environmental, social, political and individual[73]. As suggested by Dahlgren & Whitehead decades ago [74], such contours constitute a complex and dynamic network that encompasses different levels (fig.1) generally classified in three different groups according to Frohlich [75]: (*i*) *individual characteristics*, (*ii*) *physical environment* and (*iii*) *social structures and practices*. The epistemological project of Population Health as a research paradigm is therefore to investigate that web of causation and generate general causal pathways logically connecting those different levels using both theoretical assumptions, observation and empirical data. The resulting framework should also outline some key leverage points where actions can be undertaken to curve risk distribution. Health Promotionists have used the population health approach with a certain success in the recent years, but an ecological model of cancer, and prostate cancer in particular, is yet to be determined. A good candidate for contributing to such initiative is the calcium theory of carcinogenesis (Fig.2). There is a consensus that free calcium is a key player in molecular mechanism and more likely in the cancer molecular phenomenology. Indeed, the series of biological features described by Hanahan and Weineberg as the *Hallmarks of cancer*[76] are all supported by a molecular cascade in which calcium signal plays the central role. Interestingly, internal calcium

homeostasis is under the direct influence of *exosystemic* factors such as diet, life style, environmental pollutants or geographic latitude. It's therefore promising that these two theories serve as part of a framework for revisiting the complex mechanistic network of prostate cancer and its unipolar distribution in men from African ancestry. In this research, we also complement the population health perspective with the emerging notions of *intergenerational and transgenerational epigenetic inheritance* theory of cancer. In its most essential definition, epigenetic relates to the expression of cellular traits in response to environmental influences that switch the genes on and/or off [77]. The inheritable nature of those cellular changes are now surfacing in the cancer research. Proto-oncogenes and tumor suppressor genes are believed to be influenced by external environmental factors and the molecular mechanisms include DNA methylation, histone covalent modifications, nucleosome remodeling [78]. Using this framework, our research aims at providing a unifying model of prostate cancer vulnerability that would help curve the risk distribution in the most at risk populations.

Figure 1: *Dahlgren & Whitehead Socio-ecological model of health.*

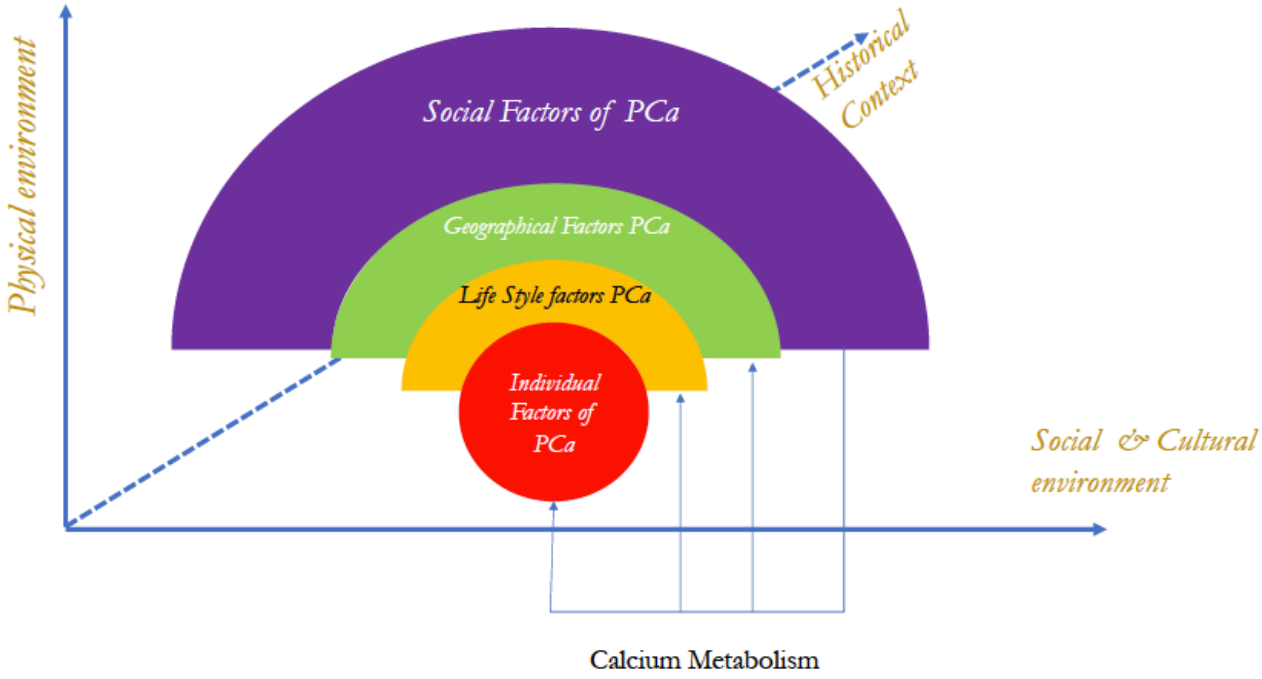

Figure 2: *Dahlgren & Whitehead Socio-ecological Model Applied to the Study.*

2. Research hypotheses

Based on the available evidence reviewed earlier, we base our research on the following assumptions :

(1) Diet is a key influencer of prostate cancer risk along with other environmental exposures.

(2) Calcium metabolism is the main pathway whereby environmental factors, including and mostly nutritional intakes, impact prostate cell lines, and this action is *mediated* by a tissue specific calcium binding protein or group of proteins.

(3) Increased incidence of the disease in males from African Ancestry derives from a conjunction of environmental factors, including new calcium intake patterns that have become heritable through transgenerational and intergenerational epigenetic and genetic mechanisms.

(4) Racial differences in outcomes, however, might be attributed more to social and material characteristics of the different male populations rather than to the disease itself.

These assumptions are best evidenced if a multi-method study is conducted at both the biological and epidemiological levels.

3. Research Design

This research uses a multi-method design. Multi-method approaches in research have been defined as an attempt to combine different scientific inquiry techniques to address a particularly complex research question[79]. The combination may include a set of different qualitative and/or quantitative approaches or even breach with the quantitative /qualitative divide to include other experimental models. In general it's indicated in three (3) instances : (*i*) when faced with weaknesses in the current approaches or data sources, (*ii*) when the study intends to capture multiple levels interactions from fields using different research paradigms and (*iii*) when the investigators aim to increase the audience of a research to trigger immediate action, say from policymakers [79]. Given the complexity and the multidisciplinary nature of our enquiry, it was deemed appropriate to deploy a multi-method approach using the most appropriate design for each research question. For methodological reasons, the study has been structured in three different phases (Fig.3): Phase I will be a critical review of the literature, Phase II an observational study, and Phase III a Proteomics research. Further details on the different study Phases are provided in the following paragraphs. The following chapters of this monograph (Chapter 4, 5 and 6) describe in full details the specific method to each phase, results and the partial conclusions, before the synthesis and discussion chapters (Chapter 7 and 8).

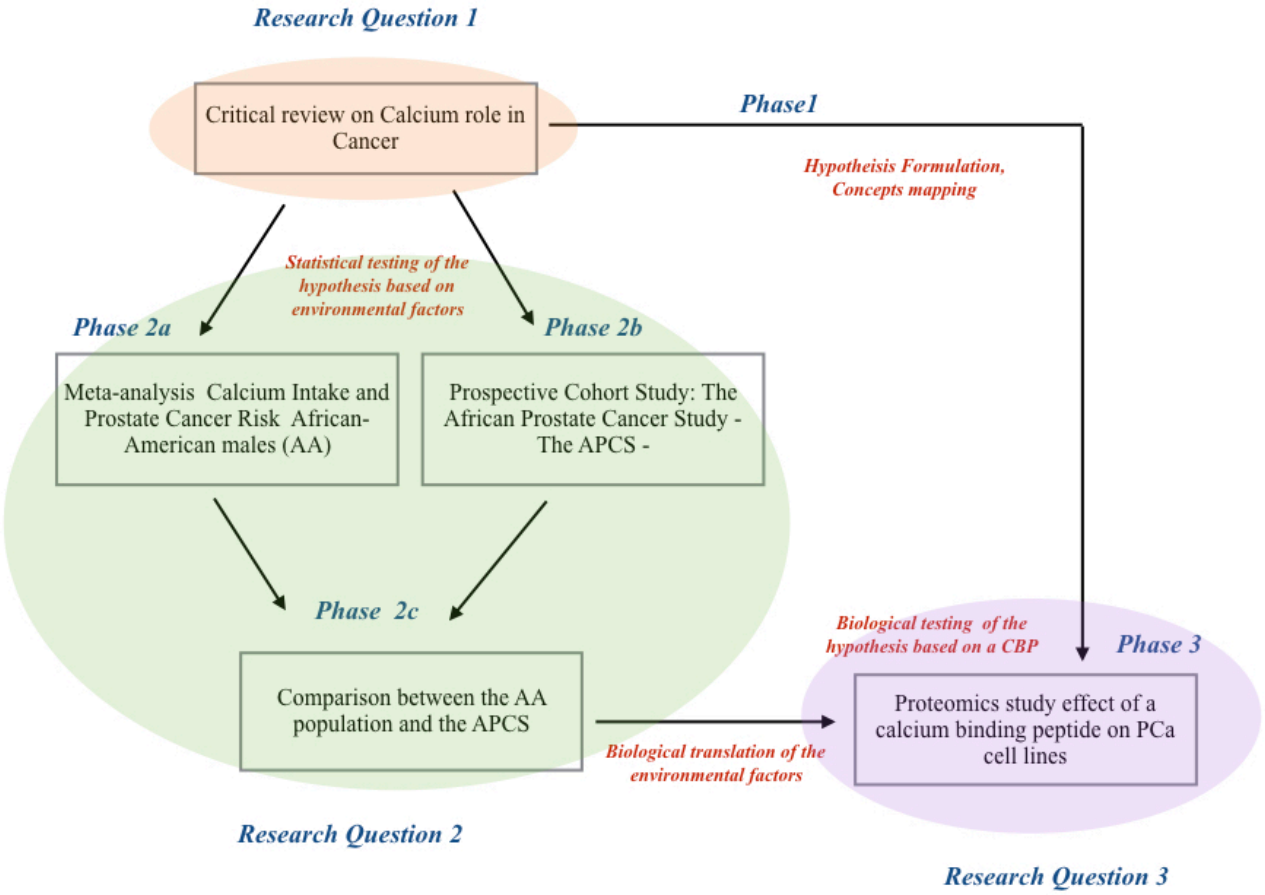

Figure 3 : Design Aspects of the Study.

Chapter 4 :Calcium Role in Carcinogenesis: A Critical Review of the Literature (Study 1)

The first study of our research is focused on *Research Question 1: To what extent the existing body of empirical evidence supports the role of calcium in cancer ? What causal mechanisms could be suggested linking prostate tumorigenesis to nutritional calcium and to other environmental calcium metabolism influencers?* As described in our introductory chapter, Study 1 has 3 objectives : *Study Objective 1*: To critically synthesize and the body of empirical evidence linking molecular calcium to prostate tumor initiation, promotion and progression; *Study Objective 2* : To identify the principal molecular mechanisms involved in calcium-centered carcinogenesis; *Study Objective 3*: To suggest a new theoretical model of cancer causation based on molecular calcium.

1. Study method

Critical reviews aim to develop, through an extensive search of the literature with their critical evaluation for quality, new models, concepts and relations between subjects of interest[80]. They are constructed through an integrative analysis based on existing data. Although the method lacks some systematicity, the emphasis is on the conceptual contribution of each item of included literature. They share some methodological similarities with integrative literature reviews and conceptual theory manuscripts. It's generally acknowledged that it's a good starting point for complex research.

1.1. Study Framework

The framework guiding the review was sourced in a functional analysis of a compartmental model of calcium homeostasis. As defined by Bassingthwaighte et al. [81], compartments in physiology are composed of sets of interconnected mixing chambers. Each component of the system being considered as homogeneous instantly mixed, with uniform concentration. The key events in the model are chemical reactions, trans-membrane transport system, and binding processes. As for calcium homeostasis, two main compartments are generally distinguished: (*i*) the extracellular compartment, which comprises *serum free calcium* or extracellular calcium, here on referred to as $[Ca^{2+}]_0$ and (*ii*) the intracellular compartment with *cytosolic free* calcium or intracellular calcium, noted $[Ca^{2+}]_i$. The latter comprises some important sub-compartments, the intracellular organelles,

which mainly sequestrate or release calcium in response to a specific signal. These are mainly the Mitochondrion (M), the Endoplasmic Reticulum (ER) and the Trans Golgi Network (TGN). An additional compartment now gaining interest in the calcium cancer literature is the Nucleus (N). Hence, we approached the different compartments focusing on the *calcium homeostasome*. The term refers to the ensemble of ultra-structures, architectures and interactions in eukaryotic cells interplaying to ensure normal calcium signal transduction[82]. It is also referred to as the calcium *signaling toolkit*. The illustration of the compartmental model is described in figure 4.

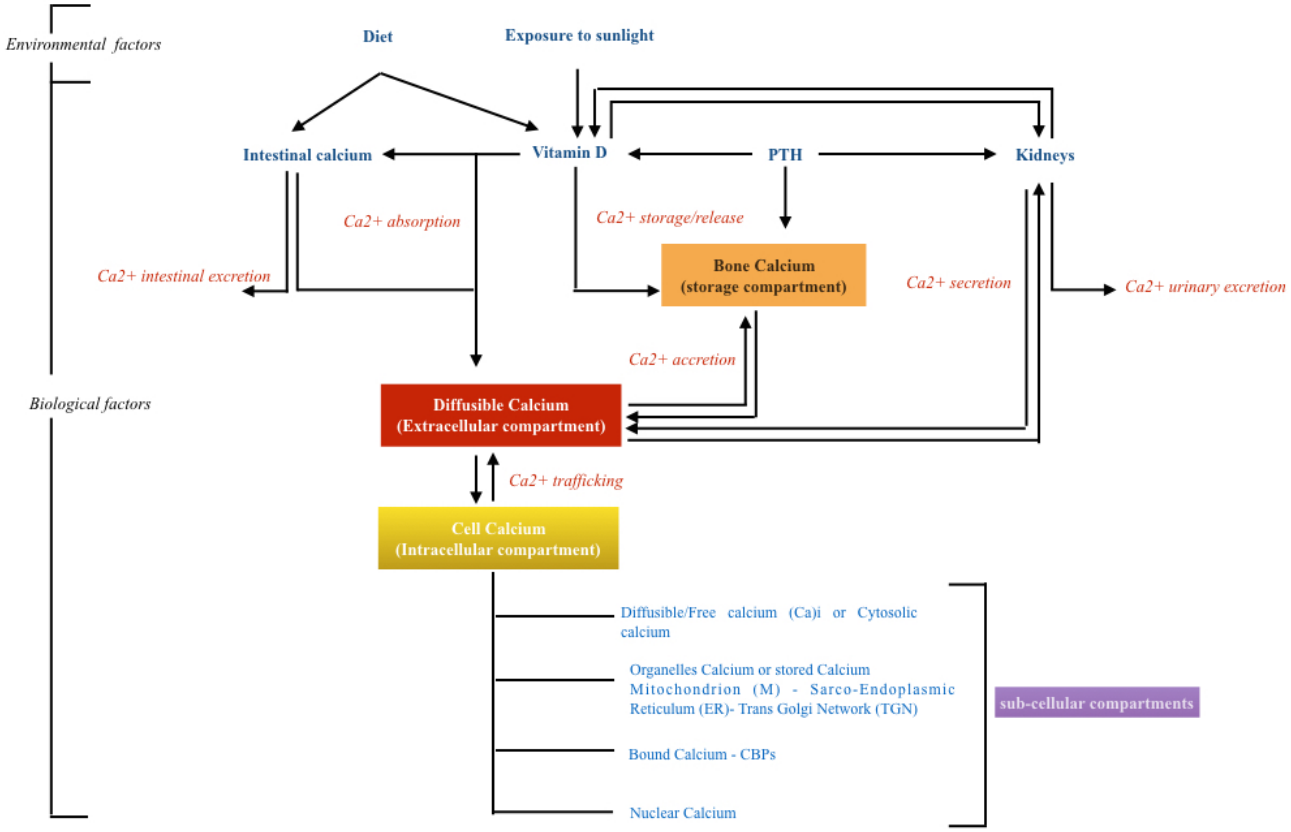

Figure 4: Compartmental model of calcium homeostasis with the different compartments.

1.2. Literature Search

A Boolean search was conducted early May 2015 on different databases including: Medline (Ovid), PubMed, ProQuest, Cochrane, Google and Google Scholar. The review included both English and French languages manuscripts, peer-reviewed and gray literatures and published from 1900 up to the end of December 2015. The *calcium and cancer literature* being dynamic and fast growing, we have added a second set of articles published in the first semester 2016. That search was complemented with a manual search based the references of randomly selected 10 articles among the most recent publications retrieved. The technique has been used before by Pham et al. in scoping review published in 2014 [83].

2. Results

A body of empirical studies or their reviews comprising 161 articles published between 1904 and 2016 were reviewed and analyzed, the key findings of this review were published in a Cancer Metastasis Review (CMR) a Springer Journal, in September 2016[1]. They were also presented at the Paris France, Cancer International Symposium in November 2016. The key findings are developed herein.

2.1 Calcium ion in biological systems

Calcium ion (Ca^{2+}) plays a central role in biological systems, due to its intrinsic chemical properties. These are best reflected when the ion is compared to its Magnesium (Mg^{2+}) counterpart, as both elements belong to the same alkaline earth metals group. In addition, Mg^{2+} is always present at concentrations varying between 1 to 3 mM in the biological fluids in which calcium performs its physiological functions. Both metal ions exhibit a strong ligand preference for oxygen (O) over nitrogen (N) and sulfur (S). But, contrarily to Mg^{2+}, Ca^{2+} is able to bind to all O atoms from biologic carboxylates groups, including even those attached to alpha-carbon. Besides, the ionic distance between oxygen and calcium varies from 2.30 A° to 2.50 A°, whereas Mg^{2+} is always at a fixed distance of 2.05 A° at the center of octahedron of oxygen atoms. As a consequence, complex biomolecules such as intracellular proteins will show a higher selectivity

[1] Cancer Metastasis Rev (2016) 35:391–411
DOI 10.1007/s10555-016-9634-0

(in the range 10^4 more stronger) for calcium. Also calcium has a comparatively low degree of hydration. Whilst the element gives a relatively simple divalent cation in water, its degree of hydration varies rapidly from 6 to 8 water molecules making of it the fastest binding divalent ion (over 10^3 times faster than Mg^{2+}) [84]. Finally, calcium forms insoluble salts with both organic and inorganic anions such as carbonates and phosphates and gives precipitates limiting its solubility around 10^{-3} M. As a consequence, hydroxyapatite [$Ca_5(PO_4)OH$] the main constituent of the bony tissue is the most stable salt at pH >5. These properties render calcium ion one of the most reactive chemical elements in biological systems and account for its central and universal roles in cellular machinery. However, there is a downside to that high reactivity, especially when we consider the additional fact that the body cannot catabolize the cation. Without any control mechanism, there is the permanent risk that calcium ions would react untimely and inappropriately with other biomolecules. In prevention to this damageable event for the whole biometabolism, a three-fold solution is utilized by the system [85] : (*i*) *Compartmentalization*: the ion is dispatched in different compartments within the system, having each a specific function modulated by a specific calcium concentration (*ii*) *Extrusion and intrusion* or *trafficking:* when necessary, Calcium ion actively traverses the membranes separating the different compartments of the system. (*iii*) *Chelation*: the ion is chemically bound to specific biomolecules following electrochemical driving forces and constrained by thermodynamic laws. The prowess here is that, binding calcium also allows the biomolecule itself to perform its own physiological function through induced conformational and/or electrostatic changes. The variations in calcium compartmental concentration are the main parameter determining the interplay of these different control mechanisms. In other words, calcium itself triggers its own released or storage mechanisms, a principle known in the calcium literature as *the calcium-induced calcium released mechanism*. And it is now established that all body cells use that regulation mechanism[82]. Bringing the compartmental concentrations of calcium ion back to its resting values is then the ultimate goal of the homeostatic mechanisms, which in the same time is the underpinning physiological rationale for calcium signaling.

2.2 Serum calcium regulation and calcium signaling

The quasi totality of body calcium derives from nutritional sources. Intestinal absorption is under the dependence of both diffusional mechanisms as well as of active mechanisms involving: Vitamin D, calcium channels such as the Transient Receptor Potential Cation Channels of the

vanilloid family member 6 (TRPV6), and carrier proteins of the Calbindin D_{9K} family. The molecule is then extruded from the epithelial intestinal cells toward the circulatory system from whence it is redistributed to the other tissues. The extrusion system is based on pump molecules, of which the CaATPase and the Na^{+}/ Ca^{2+} antiports have been more researched. Serum calcium ranges from 8.8 to 10.4 mg/dl (2.2 to 2.6 mM) under normal physiological conditions. It comprises three different fractions including free calcium [86], its physiologically active form. Calcium homeostasis at the serum level, involves a series of humoral factors, hormonal actors and effector organs in a constant cross-talk to maintain extracellular calcium ($[Ca^{2+}]_0$) in the physiological ranges. The Calcium Sensing Receptors (CaSRs) expressed by different body tissues are believed to play a critical role in that control. We'll see later in the coming paragraphs that these receptors also intervene in cancer mechanisms. In short, Calcium signaling generally refers to a cellular signal induced and transducted by the calcium ion. Its underlying principle remains similar to the one described earlier: a change in intracellular concentrations of free calcium ($[Ca^{2+}]_i$), triggers a series of reactions, the ultimate goal of which being to restore the concentrations to its resting values. Compared to the extracellular milieu, intracellular calcium ($[Ca^{2+}]_i$) is relatively low (approximately 100 to 200 nM) as demonstrated earlier by Tsien [87]. And that difference in calcium concentration between the intracellular milieu and the extracellular milieu, along with the intimate mechanisms developed to always maintain that difference, is probably the essence of living cells as stated by Williams [88] and Rizzuto [89]. The sequence of events leading to a calcium signal transduction has been extensively described in the calcium and cancer literature [90]: an initial stimulus drives calcium into the cell, thus augmenting $[Ca^{2+}]_i$. In the intracellular environment calcium will be dynamically sequestered and exchanged between different sub-compartments comprising the cytosol, key organelles, the Endoplasmic Reticulum (ER), the Mitochondrion (M), the Trans-Golgi-Network (TGN) as well as the nucleus (N) [85] . A complex system of pumps and channels participate in that trafficking. Amongst which some have been related to cancer, as we'll see later: the Sarco-Endoplasmic Reticulum Calcium-ATPase (SERCA), the STIM/Orai system as well as the Transient Receptor Channels (TRPs). While moving from one compartment to the other, the divalent cation is bound by a plethora of proteins, either for buffering reasons or for regulatory functions [85]. These are referred to as the calcium binding proteins which play an essential role in biological systems[91]. At the end of the processes, intracellular calcium is sent back to the extracellular milieu through a dynamic pumping system and the low baseline levels of the ion is therefore restored. The nature of the initial or triggering stimulus and both the amplitude and frequency of the induced

variations in $[Ca^{2+}]_i$ is basically what differentiates one calcium signal from another. Hence, core notions such as calcium waves, burst, sparks, oscillations and peaks are extensively described in various work conducted by cellular electrophysiologists [92]. As mentioned earlier, these different actors constitute the calcium homeostasome (Fig. 8). A dysfunction or disruption of this essential cellular machinery has been linked to cells cancerous transformation, promotion and progression.

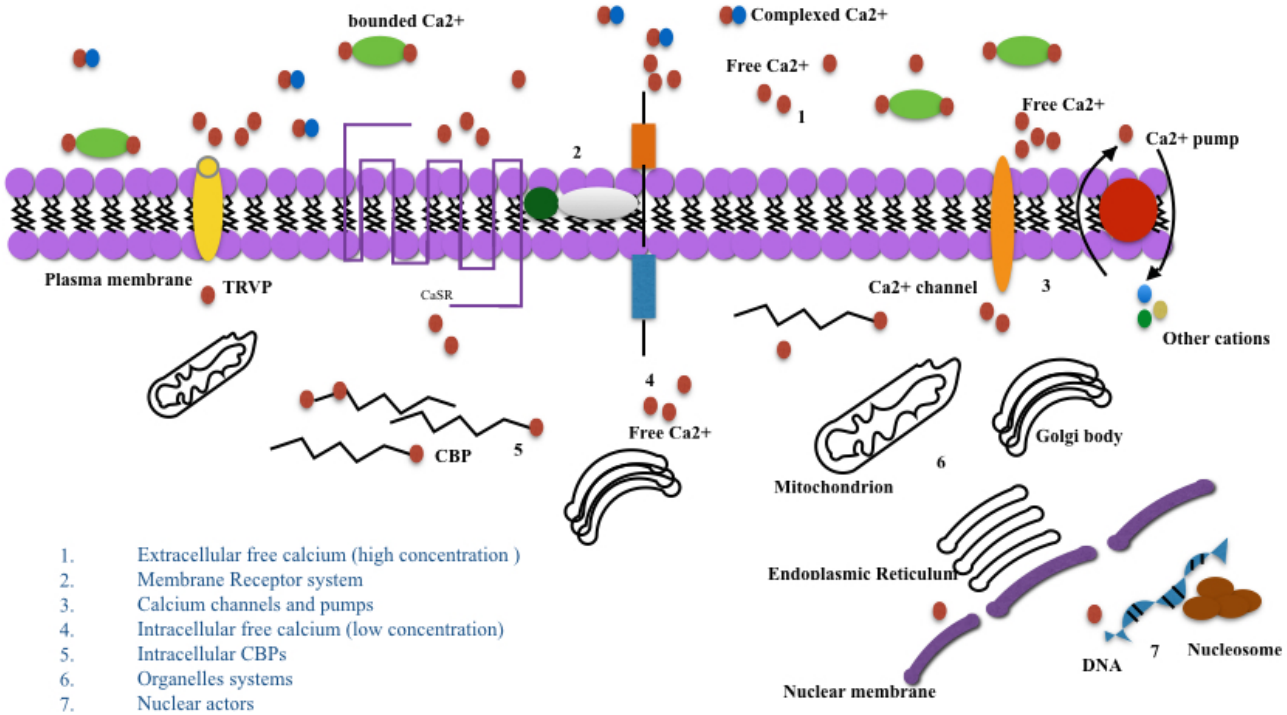

Figure 5: Elements of the calcium signaling toolkit.

2.3. Calcium homeostasis and tumorigenesis

2.3.1. Earlier studies

Research conducted in the early 1900s suggest that most cancer cells have in general a relatively low calcium content when compared to their normal cells counterpart [93, 94]. This provided the initial indication of possible connection of tumorigenesis with that cation. In earlier experiments conducted by Carruthers and Suntzeff [95], the authors measured the calcium content in an artificially induced epidermal carcinoma cells in mice. Evolution toward cancer revealed two distinct phases: an immediate reduction in calcium content persisting for many weeks, followed by a further reduction when the cells have been transformed into cancer. Such observations were corroborated by other studies using the micro incineration techniques and measuring the calcium content in benign hyperplasia lesions such as hyperkeratosis and warts, as well as in breast and skin carcinomas. The conclusion remained the same: the divalent cations such as calcium and magnesium are present in diminished concentration in either cancerous or hyperplasic cells[93, 95]. In 1977, Miller conducted a review in which three main segments of cellular activity were impaired in cancer cells and those defects were directly correlated to calcium: (*i*) reduced cell adhesiveness of cancer cells: a positive correlation between diminished calcium content and a loss adhesiveness amongst cancer cells was observed, (*ii*) increased motility of cancer cells: the authors attributed the increased in cancer cell motility to an electrostatic force induced by a diminishing of positive charges from the lack of positive cation such as calcium. (*iii*) Increased permeability of cancer cells: this would explain the leakage of proteolytic enzymes in the extracellular environment, which in turns contributes to the metastasis of the tumor. A few years earlier, Lowenstein (1975) had shown that calcium ion played a primarily role in the process of cellular communication and membrane permeability through cellular membrane junctions. A good summary of these early findings was provided by Lansing et al. [96] who suggested that, since serum calcium levels were unchanged in cancer patients (at least at an early stage of the disease), and on the other hand cancer cells demonstrated a relatively low calcium content, thus cancer would involve an alteration in a calcium-binding protein complex located at the cell surface that ultimately impeded Ca^{2+} exchange. This postulate still echoes today. In 2007, Monteith and co-workers conducted a review on targeting Ca^{2+} transport as a viable therapeutic option for cancer [97]. The authors concluded that in spite of Ca^{2+} being a ubiquitous cellular signal, available evidence support that specific channels or pump could be targeted. In the

following paragraphs we identify the specific elements or mechanisms of the calcium signaling toolkit that have been linked to carcinogenesis.

2.3.2. Alterations in cell surface calcium sensing receptors (CaSRs) expression

Various studies on CASRs assert with a certain degree of confidence their potential role in carcinogenesis. However, this role is histologically differentiated since the receptor was evidenced to work as a tumor suppressor in some tissues and as a tumor promoter in others [98]. Such versatility is not surprising per se, under normal and physiological conditions, CaSR biological function is always performed in a tissue-dependent manner[99]. However, the precise mechanisms underlying CaSRs contribution to carcinogenesis remains hypothetical. What is most consensual is that some cancer cells demonstrate a genetic alteration of CaSRs expression at the surface of their plasma membrane [98]. For example, in some cancers the overexpression of CaSRs is hypothesized to be chemopreventive, whereas in others it is rather seen as pejorative. We briefly describe CaSRs role in Colorectal cancer, breast cancer and prostate cancer.

CaRS role in colorectal cancer

In normal conditions, CaSRs are highly expressed at the surface membranes of colonocytes, the epithelial cells of the colon. Several authors account that physiological overexpression as having a protective effect against colonocytes proliferation. The core element of this protective mechanism is believed to be extracellular calcium itself, acting synergistically but independently with some endogenous amines, such as polyamine spermine [100]. Different pathways are suggested: (*i*) CASRs activation of the antitumor p38 mitogen-activated protein kinase cascade [28], (*ii*) promotion of cell-cell adhesion, by enhancing E-cadherin production [101] and (*iii*) the suppression of the β-catenin/T cell factor complex of the Wingless signaling pathway (Wnt) [102]. Thus as colonocytes migrate from the base to the surface of the intestinal crypts, they undergo progressive structural changes to reach terminal differentiation and then undergo apoptosis at the epithelial surface of the crypts. In this transit though, the epithelial cells migrate from layers low in calcium concentration, and subsequently active cell proliferation, to layers of higher calcium concentration, where activated CaSRs work as a switch to turn off the proliferative activity of the cells and trigger apoptosis.

Two additional comments could be made at this point. Firstly, some studies link the expression of CaSRs at the surface membrane of colonocytes to epigenetic mechanisms such as DNA

methylation and histone modifications. Such modifications are hypothesized to potentially contribute in silencing the CaSRs coding genes. Furthermore, recent research have identified DNA binding sites of the two Vitamin D Receptors Elements (VDREs) in CaSRs promoters, suggesting the prominent role of Vitamin D in enhancing the expression of CaSRs [103]. Much more recently, additional factors such as TNF alpha and IL6 have also been said to play a role in the transcriptional and translational activation of the CaSRs. Secondly, these molecular findings are in line with observational studies supporting the protective effect of relatively augmented calcium and Vitamin D intake against colorectal cancers[104].

CaSR role in breast cancer, prostate cancer and bony metastasis

Several published data suggest that epithelial breast cell lines demonstrate an increased differentiation when calcium is added to the growing culture medium[105]. The receptor has been localized on the surface membrane of the ductal epithelial cells, and it is likely to regulate Ca^{2+} homeostasis and milk transportation during lactation. The series of experiments conducted years ago by Liu, Hu and Chakrabarty [101] using two different breast cancer cell lines, ER+ MCF-7 and ER-MB 435 are quite informative. Extracellular calcium ($[Ca^{2+}]_0$) inhibits cell proliferation and invasion in a CaSR-dependent manner. It also increased the cytotoxic activity of chemical agents such as paclitaxel as well as reducing the expression of the antiapoptic protein, *survivin*. Here too, the two remarks raised earlier with colorectal cancer apply: CaSR expression is closely correlated to Vitamin D metabolism[101, 106] and this is supported by epidemiological studies. High dietary calcium and vitamin D intake are generally associated with a reduced risk of breast cancer as evidenced by Negri and coauthors[107] and a cohort study (2005) conducted on 68,567 postmenopausal women [108]. But there is a nuance here. The chemoprotective effect of calcium in breast neoplasm may not follow a linear relationship. Other studies suggest a more dose-dependent-like effect, based on local variations in concentration of extracellular calcium ($[Ca^{2+}]_0$). When $[Ca^{2+}]_0$ is kept within the physiological ranges, the protective effect of calcium is observed. At a certain level beyond the physiological levels (say above 20 nM), $[Ca^{2+}]_0$ increases estrogen receptor (ERs) transcriptional activity [109]. Indeed, a weak estrogenic effect was observed when local extracellular calcium increased beyond a certain level. That local increase in calcium levels is also accounted for the metastatic potential of breast tumor. One suggested mechanism relies on the local production of a protein, the PTH related Peptide (PTHrP). It has been reported that several other cell types secrete PTHrP in response to CaSR activation. Amongst these are prostate cancer cell lines, breast cancer cell lines, and rat Leydig

cells. The protein exists in three different isoforms, of which isoform 1-139 has been recognized as the most active[110]. Experimental studies support that PTHrP secreted by the primary tumor under CaSR activation, develops an osteolytic activity through an increase in bone resorption[110]. In the bone microenvironment, the peptide acts synergistically with a growth factor, the Transforming Growth Factor-Beta (TGF-β). The increased bone resorption is responsible of a subsequent increase in extracellular calcium concentrations, classically known as the humoral hypercalcemia. In turn, the hypercalcemia activates the CaSR which also lead to further secretion of PTHrP ultimately leading to more bone metastasis in an auto-perpetuating mechanism. Thus CaSR plays a significant role in bony metastasis in breast cancer through an increase of local extracellular calcium beyond 40 nm[109]. A similar role of extracellular calcium through the CaSRs pathway as a main contributor to tumor progression and bony metastasis is described in prostate cancer cells as well, as supported by experiments conducted on different cell lines including LNCaP, C4-2B and PC3. Two remarks could be elicited from those different findings: (*i*) increased $[Ca^{2+}]_0$ stimulates prostate cancer cell growth in all three (3) cell lines; (*ii*) reduced expression of CaSR limits PC-3 proliferation and suppresses tumor progression. Although different well known pathways could be involved such as Akt and ERK pathways, PTHrP appear to be the main bony metastasis promoter.

2.3.3. Alterations in transmembrane calcium trafficking proteins expression and cancer

In their review published in 2007, Montheith et al. argued against the general idea that calcium was too universal a cellular actor to be considered a good therapeutic target in combatting cancer. They noted that the cancerous transformation was constantly associated with a change in expression of some key membrane-bound proteins involved in the transcellular calcium trafficking[110]. That argument was further reinforced by various knockout experiments, targeting the coding genes of those proteins. An experiment conducted a decade ago by Prasad and co-workers directly connected changes in Sarcoendoplasmic reticulum calcium-ATPase (SERCA) expression and initiation of squamous cells carcinoma in mice [111]. In this section we will briefly focus on alteration in the expression of two groups of structural proteins which have been shown to be actively involved in Ca^{2+} transmembrane trafficking : the Transient Receptors Channels (TRPs) and proteins pertaining to the Store Operated Calcium Entry (SOCE) system. As these proteins are related to the entry mechanisms, we will also mention the role of Sarcoendoplasmic Reticulum Calcium-ATPase (SERCA) on the extrusion versant.

2.3.4. The Transient Receptors Potential Channels (TRPs)

Like the calcium sensing receptors reviewed above, the role of TRP channels in carcinogenesis is versatile. They appear as oncogenic in some tissues, and rather as chemoprotective in others, depending on their *local role* in calcium signaling [112]. Like the CaSR again, that carcinogenic role in most cases, is related to a quantitative change in the channel expression at the plasma membrane surface. Rarely a somatic genetic mutation is involved[113]. The most studied TRPs in regard to cancer to date are the subtypes TRPM8, TRPV6, TRPM1 and TRPV1. They are strong candidates for cancer biomarkers and even for therapeutic targets [49, 50]. How the expression of these channels are deregulated is now known although partially. Three types of defects are commonly described: (*i*) transcriptional and translational modifications, (*ii*) impairment in their localization at the plasma membrane surface and (*iii*) perturbation in their intrinsic activity. As for the underlying mechanisms for theses defects, perturbations in hormonal activity is suspected especially for TRPM8 and TRPV6 [114]. This is well supported by experiments conducted in human hormone-dependent malignancies such as prostatic or mammary adenocarcinomas, where theses channels are over or under expressed [113] Furthermore, the overall mechanism, as evidenced by different research, has been described. Under the influence of androgenic hormones, and/or of some growth factors such as the Hepatocyte Growth Factor (HGF), changes in the expression of TRP channels, and particularly TRPM8 and TRPV6 occur. This happens through a direct influence on their nuclear synthesis processes, on their plasma membrane localization and on their intrinsic activity. The common consequences of such changes is a reduced uptake of free calcium from the extracellular milieu, leading to a subsequent decrease in $[Ca^{2+}]_I$. The subsequent *intra cellular hypocalcaemia* alters in turn internal calcium signal, especially in cell cycle regulation and in apoptosis, ultimately leading to a diminished or loss of control mechanisms over cell growth and proliferation [115].

2.3.5. The Store Operated Calcium Entry (SOCE) system (STIM1/ORAI) and the SERCA pumps.

Another segment of the calcium signaling toolkit, which is now being investigated, is the store operated calcium entry (SOCE) mechanisms and their potential role in carcinogenesis, especially in cell proliferation, apopto-resistance and metastasis. In short, the SOCE is a mechanism whereby additional free calcium is driven into the intracellular compartment when a subcompartment, such as the ER, *sees* a decrease in its initial store of calcium. It is based on two interacting membrane proteins the Stromal Interacting Molecule (STIM) and the ORAI protein

coded by the Orai gene [116]. An upregulation of the SOCE system is believed to be a substantial contributor to carcinogenicity [117]. Two pathways are suggested : the first is based on a dynamic interaction between the STIM/Orai system and other calcium dependent effectors such as calpain, Pyk2 phosphorylation, Ras & Rac1 activity. The second mechanism focuses on a deregulation of cell cycle regulators such as p21 and Cdc25C, leading to an enhanced endothelial cell proliferation, angiogenesis and subsequent increase in the secretion of Vascular Epithelial Growth Factor (VEGF) [118]. The ensemble ultimately facilitates tumor progression. Indeed, silencing experiments conducted on the STIM1 or the Orai1 genes have lead to an inhibitive effect on breast cancer cell lines migration. Quite a similar response was also obtained using hepatocarcinoma cells and human cervical cancer cell lines[118] . Along with the vascular processes, focal adhesion turnover and actomyosin contraction were also believed to contribute to cancer cells migration[117].

On the extrusion versant of the calcium signaling toolkit, the Sarco-Endoplasmic Reticulum Calcium ATPase (SERCA) pumps have also been proved to be actively involved in both tumor promotion and progression. Some experimental drugs are under development targeting the SERCA [119].

2.4. The role of non-membrane-bound calcium binding proteins (CBP)

2.4.1. The S100 family

The S100 family is a EF-Hand type group of 26 calcium binding proteins (CBPs) sharing in common a 100% solubility in saturated ammonium sulfate at neutral pH[91]. A deregulation of S100 proteins expression is commonly observed in most human cancers [120]. No less that 10 members of the family has been experimented as key contributors to tumor development, from growth to metastasis. A review published a few years ago by Mishra et al. on the S100A4 member of the family gathered clinical evidences relating the molecule to a various types of cancers. The list was quite significant and varied from common digestive cancer (gastric, pancreatic, colorectal) to glandular neoplasms (thyroid, breast, prostate) including other organs such as the kidneys and the lungs cancer. The molecular mechanisms underpinning the action of S100A4 was based on interactions with molecules including p53, cytoskeletal proteins, and a certain role in the degradation of extracellular matrix[63]. There is a growing consensus that S100 proteins could serve as specific cancer signature as they exhibit a certain profile according to the type and stage of a given cancer. Clinical research come in support with the ongoing trial

targeting S100B in melanoma and S100A8 in prostate cancer[121]. The latter has been shown to inhibit the telomerase activity [122]. Such a signature is already available for certain neoplasms such as for head and neck, prostate and colorectal[123] cancers. Furthermore, exception made for two cancers, it is reported that the typical deregulation of S100 family expression commonly observed in cancers is an upregulation. However, their specific mechanisms whereby S100 family is over expressed in human carcinogenesis remains unclear. An interaction either with matrix metaloproteases or with p53 is highly suspected by some authors[124]. An emerging current of thought on the subject focuses more on epigenetic mechanisms as DNA methylation. For example, DNA methylation is reported to regulate S100P expression in prostate, pancreatic and cervical cancers; S100A4 expression in pancreatic, endometrial, gastric, breast, ovarian, renal and brain tumors; S100A2 expression in prostate and breast cancers [125]; S100A6 in prostate and gastric cancer and S100A10 in the pituitary gland cancer[126].

Also, it is to be noticed that different signal transduction pathways could interplay in the same cancer. In colon cancer for example, S100A4 expression is controlled by WNT-Beta catenin signaling, whereas S100P expression is regulated by activation of both the prostaglandin E2 (PGE2) receptor EP4 and the CREB pathway [127]. Finally it is noteworthy that S100A7 and A4, A8 and A9 could serve as better biomarkers for breast cancer for example when compared to the currently available methods [128].

2.4.2. The granin family

Granins are a family of 5 calcium binding proteins found in the cores of peptide or amine hormones as well as in the neurotransmitter secretory vesicles. They are markers of the neuroendocine cells[128]. Hence their role has been researched in hormono-dependent cancers in general, and in the prostatic adenocarcinoma (PCa) in particular. Secretogranin II (SgII) is over expressed in advanced PCa and the protein was identified as the main promoter of the neuroendocrine differentiation observed in the hormone-resistant form of the disease. Neuroendocrine differentiation (NED) here relates to the presence of scattered neuroendocrine cells, singly or in small nests, in conventional prostatic adenocarcinomas[129] . NED is believed to account for PCa resistance or relapse after the conventional treatment. Also, Chromogranin A (CgA), secreted by NE cells, is believed to be more specific to and more informative on the stage of PCa than the classic PSA [130].

2.4.3. The proprotein convertases family (PCs)

Proprotein Convertases also known as PCSKs (Proprotein Convertase Subtilisin Kexin type) are a family of CBPs that activate other proteins[131]. Furin has been the most researched member in the family in regard to tumor regulation, promotion and progression[132, 133]. In normal tissues, Furin is typically detected at very low concentrations. Conversely, overexpression of the protein's mRNA is observed in several tumors. Even more, the aggressiveness of a given tumor is positively correlated to plasma levels of furin[35, 134]. We mentioned in the earlier paragraphs the detrimental role played by PTHrP in promoting bony metastasis. Furin's activity is also enhanced by the peptide, an action to which other calcium dependent elements of the tumor microenvironment such as BCl2 contribute[135]. The Transforming Growth Factor Beta (TGFβ) mediates the role of Furin in tumor promotion and progression, as supported by different research [136]. Additional pathways include the well known RAS-ERK pathway as well as the Hypoxia-Inducible Factors (HIF).

2.5. Calcium and cells resistance to apoptosis

Apoptosis, or programmed cell death, first described by Kerr (1972), is a central mechanism whereby biological systems regulate tissue development and homeostasis[137]. The process is also one the most potent mechanisms against cancer. It is now well grounded that most of the cell death mechanisms are regulated by calcium [138]. In the series of events characteristic to the apoptotic machinery, the equilibrium of calcium exchange between the Endoplasmic Reticulum (ER) and the Mitochondrion (M) appear to be of prime importance. A depletion of ER calcium ore more precisely, an increase in the release of the cation and its subsequent increased uptake by the M results in the series of morphological changes that are typical to the programmed cell death. The uptake of calcium by the M. is physiologically regulated by a series of proteins situated in both the outer and the inner membranes of the mitochondrion (OMM and IMM)[137]. Apoptoresistance develops when cells escape to that control mechanism. Based on Lymphoma and Leukemia models, a protein has been identified in the IMM, the B-cell lymphoma/ leukaemia-2 (Bcl-2) which renders cells insensitive to apoptotic stimulus by reducing the filling of the mitochondrion in free calcium . In other words, an over expression of the Bcl-2 gene is one of the underlying cellular mechanisms of apoptoresistance at least in lymphomatous and leukemia cells. In some very recent research, the tumor suppressor gene p-53 has also been linked to apoptosis in a calcium dependent manner. Based on live imaging in mice cancer cells, Giorgi

and coworkers were able to assert that p-53 increases the calcium crosstalk between ER and M and thus triggers apoptosis [15].

3. Calcium-based epigenetic theories of carcinogenesis

The calcium hypothesis of carcinogenesis relies essentially on an epigenetic approach to the disease as opposed to the classic genetic mutational approach. Proponents of the epigenetic theory argue that the different somatic mutations are downstream consequences of DNA modifications[139]. Three complementary lines of epigenetic mechanisms are generally suggested: the covalent modifications of the histone protein forming the histone code involving chemical reactions such as methylations, acetylation, phosphorylation and / or ubiquitination The later lead ultimately to chromatin or nucleosome remodeling and to DNA methylation. In regard to carcinogenesis, two dominant epigenetic theories are suggested : tissue disorganization, cancer is initiated by a breakage of the intercellular gap junctions[140]; genomic catastrophe cascade, ultimately activating proto-oncogenes and /or inactivating tumor-suppressor genes [141]. Given its ubiquitous role in cancer as reviewed in the preceding paragraphs, calcium is an adequate candidate to reconcile these different views. And this becomes more evident bearing in mind that cancerous transformation is generally the result of a chronic cell injury of multiple etiology . Several arguments deriving from both experimental and clinical research support such an approach.

3.1. Increased compartmental calcium concentration initiates cancerous transformation

Earlier experiments conducted on different cell lines pointed to the role of extracellular calcium in cancerous transformation. Consistent experiments conducted decades ago support that a reduction of extracellular calcium in the growth medium under a certain value had an inhibitive effect on cell's cancerous transformation[142]. Furthermore, such a transformation was also inhibited when the cells were treated with calcium channels blockers, indicating that it needed to enter into the cells nucleus to produce the effect. It is well established that tumor-inducing viruses, such as HLTV synthesize typical proteins namely HTLV-1p13 and HCVp7, shown to increase cytosolic calcium through a release from the mitochondrial stores. Conversely, they also secrete two other proteins, HIV VPR and HBV VP which promote cell death via apoptosis [143]. It can rightly be anticipated that increases in compartmental concentrations of calcium may

initiates carcinogenesis via some cytoplasmic and / or cytosolic effects as well as some nuclear effects.

3.1.1. The cytoplasmic effects of high intracellular calcium ($[Ca^{2+}]_i$)

High and uncontrolled levels of intracellular calcium generally lead to cell death via different mechanisms such as necrosis, apoptosis, caryolysis or autophagocytosis[138]. However, if dying cells do not develop into cancer, the fatal increase in free calcium induces a *calcium wave* that would propagate and ultimately affect the *distant* surviving cells. « Distant » in this context includes both time, unknown delays after the lethal exposure, and space, away from the injured cells[139]. It is conjectured that the surviving cells will react to the increase in free calcium by a protective closure of the gap junctions. But as the injury perpetuates, hence the notion of chronicity, a disruption of the gap junctions and subsequent uncoupling of the cell and tissular disorganization will result as a consequence. This mechanism is described as the *« cork » gating hypothesis* [144]. If this hypothesis was verified it would fuel Sonnenschein and Soto epigenetic theory mentioned at the beginning of this section: the disclosure of cellular junctions as the underpinning dysfunction of carcinogenesis.

3.1.2. The nuclear effects of high intracellular calcium ($[Ca^{2+}]_i$)

If the cell survives to the untimely increase of $[Ca^{2+}]_i$, available research support that the nucleus compartment could be impacted and, the nucleosome would undergo some transformations. The activity of *histone deacetylase-5*, an enzyme involved in the covalent modifications of the histone tail is influenced by CBPs such as calmodulin and calcineurin and several others of the calmodulin-dependent protein kinases (CaMKs) and phosphatases families [145]. This is complemented by additional work supporting the indirect role of these two calcium dependent enzymes in regulating phosphorylation and dephosphorylation of some nuclear transcription factors, in response to an increase in intracellular calcium[146]. Furthermore, over a decade ago a novel EF-hand CBP, was identified, namely the Downstream Regulated Element Antagonist Modulator (DREAM). The CBP directly represses transcription from the early response of the proto-oncogene *c-fos* [147]. Thus DREAM is the first known CBP to function *directly* as a DNA-binding transcriptional regulator. Additionally, *in vitro* experiments have been reported supporting the effect of CBPs in nucleosome modeling in grounding the notion of *nuclear second messenger*[148]. Finally it is noteworthy that other proto-oncogenes such as *c-myc* or *c-fos* could also be directly activated by increased nuclear calcium[149]. The direct or indirect action of high

calcium on proto-oncogene supports the genomic catastrophe scenario hypothesis enunciated in Schachaf epigenetic theory.

3.2. Habituation of cells to lower levels of calcium

As mentioned above, distant cells react to a steady increase of calcium wave through a protective closure of gap junctions. Beside the risk of breakage of these junctions as the increase perpetuates, there is another downside to that protective mechanism: the cells lowers their dependence *vis-à-vis* calcium. Over time they *habituate* to lower cytosolic free calcium levels and they escape to all growth and proliferation control mechanism. One can conjecture that such an evasion could be related to the fact that most of these control mechanisms are mainly calcium dependent, thus less calcium in the milieu would ultimately mean less control[150, 151].

4. Synthesis, Conceptualization and Integration

What coherent sense and meaning can be made from all these accumulated observations, experiments and prior theories? In a seminal work published over a decade ago, Hanahan & Weinberg (2000) anticipated that the most significant change that may occur in cancer research for the next generation would be rather conceptual than technical. In their perceptive, the apparent complexity of the disease will be broken down unto a small number of underlying principles that would make the whole science of cancer more logical[76] . It is obvious that the entire field will benefit from such a conceptual simplification. However, for this to happen, an in-depth analysis of already known cancer pathways is necessary in order to identify some logical relationships between them beyond their traditional crosstalk. In addition, new pathways should be sought based on criteria such as integration and simplicity [152]. An attempt for such an integration was provided by the work of Kessler, Hache and Wierling, in which the authors use a top down approach to form a module-based network of cancer relevant signaling pathways [153]. As mentioned in our introductory paragraph, this type of approach would help building a more coherent and comprehensive schematic presentation of cancer physiopathology. It appears through this review that the current state of the knowledge on calcium role in cancer, allows a positioning of the divalent cation as a serious candidate on the way toward such conceptual revolution. In the second section of this paper, it has been clearly evidenced that quantitative alterations of the expression of different members of the calcium signaling toolkit was observed

in most cancer types, but in a tissue dependent-manner. In other words, for a given tissue, the expression of a specific set of signaling molecules was quantitatively altered, more frequently in the sense of an *over-expression*. Then the question logically emerging from such an observation becomes: are those expressional modifications inferential for the cancer teleology or they are rather its consequences? In the third section, we gathered evidence supporting that from an epigenetic perspective, gene expression could be altered through the direct action of calcium fluxes on the nuclear compartment. It was also evidenced that there is an overall protective response of the cell to unexpected changes in calcium concentrations and fluxes. In this section, it seems appropriate to correlate these evidences to already known cancer pathways and to envisage the possibility of a new cancer pathway.

4.1. Calcium homeostasis and cancer pathways

As critical as is its role in biological systems [88], calcium ion is literally omnipresent in cancer related molecular mechanisms. It appears that two distinct but not mutually exclusive mechanisms relating calcium homeostasis to cancer pathways can be outlined throughout this review. The first is pathways that are *calcium-store dependent* and, the second are rather *calcium-influx* dependent. It is evident that there is a constant crosstalk between the two pathways and that they might even have some common final downstream regulation factors at the nuclear level, although calcium ion is capable of acting directly by itself.

4.1.1. Calcium store-dependent cancer pathways

What we have classified as the *calcium store-dependent pathways* are cancer pathways in which intracellular stores of calcium and their releasing and storing organelles (S/ER, M, TGN) play a critical role in conjunction with other substructral actors including membranar, cytoplasmic, or nuclear molecules. We'll briefly mention some of these pathways abundantly commented elsewhere in the cancer molecular mechanisms literature. Our first example of a cancer pathway that could be classified in this group is the *Wnt signaling pathways* mentioned in section 2. Three different pathways are described under the Wnt signal transduction mechanisms: *(i)* the *Wnt* canonnical (Wnt/β-catenin) and the Wnt noncanonical with two distinct pathways: *(ii)* the *Wnt/ Ca2+* and *(iii)* the *Wnt/planar cell polarity*. It is established that the canonical Wnt pathway leads to regulation of gene transcription whereas the noncanonical planar cell polarity pathway regulates the cytoskeleton. The noncanonical Wnt/calcium pathway regulates calcium inside the

cell [154]. In a recent work, the pre-assumed classic independence of the three signaling pathways has been challenged. Based on experiments conducted on PC3 prostate cancer cell lines, Thrasivoulou and co-workers identified six (6) Wnt ligands capable of triggering intranuclear calcium release. The release induced changes in membranar polarization of nucleus allowing the translocation of β-catenin through the nuclear pores. Thus the author suggested a *convergent model* of the Wnt signalling pathways in which Ca^{2+} and β-catenin may act in a coordinated, interdependent, rather than independent, manner[155]. The MAPK/ERK pathway represents our second example of calcium store dependent pathways. Whilst the ERK cascade is centered on adding a phosphatase group by a set of proteins to their neighboring proteins upon binding of an external ligand, available data support that high intracellular calcium concentrations either activate or, less frequently, inhibit the series. Different molecular mechanisms are proposed such as (*i*) activation of the calcium-affected kinase PYK2 [156] (*ii*) stimulation of RAS-GRF[157], (*iii*) inhibition of Ras-GAPs [158] and (iv) activation of calmodulin kinase I and II [159]. In addition, at the nuclear level, available research also suggest that elevated $[Ca^{2+}]_i$ prevent the nuclear translocation of the ERKs while reduced concentrations act as an accelerator. This action has been related to changes in binding affinity of ERK2 with the nuclear pore NUP153 which, in turns, blocks the penetration of the ERKs through the nuclear pores[160]. Furthermore, it was also evidenced that calcium contributes in the subcellular localization of the ERKs through a modulation of protein-protein interactions. Thus calcium internal stores appears as determinant to the ERK cascade. But, probably the most illustrative of the *calcium store-dependent cancer pathways* remains the STIM-Orai pathways, which have been already described in full length in the second section of this manuscript. Additional details on the pathway and its interactions with calcium stores are available in references [161]. Mention can also be made of the embryonic development cancer pathways such as the *Notch* and the *Hedgehog pathways*. For the first, experiments conducted on the different subunits of the *Drosophila* Notch, both extracellular (N^{EC}) and transmembrane (N^{TM}), revealed an influencing interaction with calcium ion. When the N^{EC} and N^{TM} were treated with the calcium chelator EDTA, a disruption of the non-covalent interactions between the two different subunits was observed. At the opposite, addition of millimolar calcium was proved to stabilize that interaction. The experiment led to the conclusion that extracellular calcium depletion was central in activating and dissociating the heterodimeric Notch Receptors[162]. Furthermore, it is also well known that the EGF repeats situated in the subunits and in Notch's ligands, selectively bind calcium ions [163]. In fact, it is perfectly arguable, as suggested by Raya et al., that the whole

Notch pathways can be viewed as an embryonic sensor for extracellular calcium levels during the left-right differentiation of vertebrates [164]. Similarly, the Hedgehog pathways has been evidenced to be dependent on both calcium stores and influx for decoding the Sonic hedgehog (Shh) signal during the embryonic development of the spinal cord [165]. Although the extent to which the influence of calcium on those two last pathways in carcinogenesis remains to be more explored, one can expect a central role given the key importance of calcium in early development and in embryonic processes. In total, available data fuels the argument that molecular mechanisms of key cancer pathways can be dependent on either extracellular or intracellular calcium stores. Thus, in regard to calcium, it is appropriate to classify them as *calcium store-dependent cancer pathways*. If verified, this novel hypothesis paves the way to a conceptual change in cancer pathophysiology.

4.1.2. The calcium flux-dependent cancer pathway

If the research conducted in the recent decades clearly elicited the role of intracellular calcium stores in tumorigenesis, it also appears now that there is a cancer pathway directly related to *calcium fluxes* across the different cellular compartments. This is what we refer to as the *calcium flux-dependent pathway,* or the *calcium signaling pathway* or the *calcium wave pathway.* It aligns with the circuitry of the calcium wave, from the extracellular milieu to the nucleus, across the plasma membrane, the cytosol and the nuclear membrane respectively. It can rightly be hypothesized that this pathway mediates the connection between the external/environmental factors and their subsequently induced nuclear interactions and changes, leading to the cancer cascade. In other words, this pathway might account for the epigenetic silencing of some tumor suppressor genes and/or for the activation of certain oncogenes as discussed previously. Key actors of this pathway include: (*i)* the calcium primary and secondary influx and efflux systems: calcium channels and pumps both membranar and cytoplasmic, *(ii)* free calcium itself ($[Ca^{2+}]_I$ and $[Ca^{2+}]_0$) and its induced calcium wave and, *(iii)* nuclear CBPs such as the calmodulin-dependent Kinases (CAMK), the phosphatases and certainly some other transcription factors of which the DREAM protein mentioned in section 3 is certainly an illustration. In this perspective, the calcium ion upgrades its position, moving from a second and third messenger as commonly seen in the classic cancer pathways, to become *its own messenger.* What distinguishes here a cancer type from another is the nature and type of channels and pump initially recruited, which are themselves a function of the initial stimulus, in terms of its physico-chemical nature and properties, its frequency and amplitude. An abundant literature dating from the beginning of what

Carafolli calls the *calcium signaling saga* [166] supports the existence of a calcium signal pathway to cancer but was never clearly formulated. Three recent works provide strong support to this hypothesis. In a research published in 2010, Feng and co-workers [167] investigated the role of the Secretory Pathway Ca2+-ATPase (SPCA) in mammary tumors. SPCA is a cytoplasmic ATP-powered pump, the role of which is to deliver Ca^{2+} and Mg^{2+} in the Golgi lumen, for protein sorting, processing and glycosylation. The authors used a range of techniques including RT-PCR, measurements of basal cytoplasmic calcium levels and topographic monitoring of calcium localization. It was evidenced that the isoform SPCA2 was highly regulated in breast cancer, and though mediated by the Orai1 protein, the oncogenic activity was independent from the pumping function (store influenced) of the molecule. While its N-Terminus interacted with Orai1, the C-Terminius of SPCA2 was shown to activate Ca++ influx into the cell. It is that deregulation of calcium influx that, according to the authors, lead to inappropriate secretion and accumulation of calcium ion, resulting in microcalcification, a radiographic "signature" in breast cancer diagnosis. The author concluded that the deregulation of SPCA2 activates a cancer pathway based on calcium signaling mechanisms in which Orai1 mediates the s*tore-independent* Ca^{2+} oncogenic influx. The authors also suggested that that pathway was druggable, since calcium blockers could potentially act on SPCA2. Beyond fueling the conceptual dynamic of a calcium signal dependent cancer pathway, the work also provided a logical explanation for tumor-related calcification, a radiographic feature commonly seen as of a pejorative significance in cancer diagnostic and prognosis, to the point where there is ongoing therapeutic research focussing on those calcifications to identify and destroy cancer cells in certain tumors[168]. Supporting the druggability of the calcium influx pathway, a very recent and quite informative work has been published by Raynal et al. and deserves a detailed attention. The aim was to identify new epigenetic drugs against cancer amongst the already existing therapeutic arsenal. The work consisted in screening over 1100 FDA-approved drugs and was based on experiment conducted with YB5 cancer-cell lines deriving from the colon cancer cell line SW48 [169]. The goal was to reactivate silenced Tumor Suppressor Genes (TSGs). In YB5-cells, the GFP gene, used as a reporter gene in the cited experiment, behaves similarly to endogenous TSGs silenced by the classic epigenetic mechanisms. Traditionally, the GFP gene can be reactivated by treatment with DNA methylation inhibitors as well as with histone deacetylases (HDAC) inhibitors[170] . The HCT116 cell line, another colon cancer derived culture was used as a control. Eleven (11) of the drugs tested demonstrated a positive hit and were validated for reactivating the silenced GFP in YB5. These were classified in 3 groups: the already known cancer epigenetic drugs were

confirmed: decitabine, azacitidine and vorinostat. A second group encompassed the cardiac glycosides such as: ouabain, lanatoside C, digoxin, digitoxin and proscillaridin A. The last group was more diversified and comprised antibiotics (oxyquinoline), disulkfiram and the anticancer drug, arsenic trioxyde. Although reactivation of GFPmRNA was observed after 24h of treatment by the 11 drugs, as well as the stress response gene p21, no changes was noted in the classic epigenetic pathways were noted. At the opposite, an increase in intracellular calcium fluxes was induced by 9 of the 11 drugs, in line with the traditional mechanism of action of the cardiac glycosides validated by the experiment. A series of subsequent tests focusing on cell calcium trafficking was then conducted which led the researchers to conclude that calcium signaling through CaMK was essential in gene reactivation. Confirming the idea that calcium signaling is a cancer pathway *per se.* Additionnal insights on the calcium influx pathway is provided by Sung and co-workers[171]. Their research focused on the TRVP6 channel and calcium influx and their interaction with the endocytic Numb1 protein in stabilizing the TSG p53. The role of calcium influx in bony metastasis has also been suggested[172]. The extent to which these conclusions could be generalized to all cancer types remains to be further explored, but at this point it is clear and evident that there is a *calcium influx pathway* to initiation, promotion and development of the most common neoplasms. Such a classification of cancer pathways in two types, in regard to their relationship to calcium homeostasis (*store* dependent versus *influx* dependent) can even be further integrated: based on what is known on normal calcium signaling, it seems that the calcium influx dependent pathway has precedence over calcium store dependent pathways. Changes in calcium influx are later followed by changes in calcium stores, which in turn influences calcium influx, creating a feedback cycle. Hence, the possibility of a common ancestry for main cancer pathways should be considered and further investigated. And it appears that alterations in calcium influx would be the best candidate for that role.

4.2. Conceptualization

4.2.1. Basic principle

Of all the body minerals, calcium is known to be central to key biological events such as muscle contraction, secretion, signal conduction and normal development. As mentioned earlier, controlling calcium through the sequence: *intrusion sequestration*, *chelation, release and extrusion,* appears to be the origin, essence and fate of all living cell[88]. The molecule has been appropriately referred to as *the life and death signal* [173]. On the other hand, one of the

hallmarks of cancer, as recalled by Hanahan & Weinberg, is *continuous growth and immortality* or an evasion from life control mechanisms [76]. Therefore, what if cancer was just a perversion of some normal calcium related processes in the regulation of life cycle mechanisms? Why should that divalent cation not prove to be the central control of carcinogenesis?

4.2.2. A suggested general molecular mechanism whereby calcium might trigger carcinogenesis

Based of the preceding sections, a calcium based molecular mechanism of cancer can be suggested. In the context of a chronic and unexpected increase in calcium influxes into the cell, mostly but not only, due to external environmental factors, cellular death will generally occur[86]. However, surviving cells would traverse two phases: an initial phase characterized by a sublethal increase in calcium influx into the cell, which can be termed as *the increase phase.* The cell logically responds to the aggression by deploying a battery of protective measures aimed at *(i)* bringing the cytoplasmic concentration of calcium back to its resting level and *(ii)* circumscribing the potentially detrimental raise in calcium to one cell by uncoupling it from the others. As the causal event perpetuates, it seems that the cell habituates to low intracellular calcium, which could be referred to as the *habituation phase.* That second phase later becomes problematic *per se*, as the intracellular physiological concentrations of calcium and probably of other related minerals, decreases below their normal values on a chronic basis. In the same time calcium ion accumulates in the immediate external environment of the cell due to the protective closure and uncoupling induced by the first phase. It appears that a significant portion of the cancer phenomenology can be tracked back to those two phases, as their consequences will affect all the cellular compartments. At the nuclear level, which is probably the first to be impacted, epigenetic modifications of both nucleosome and DNA molecule will occur, ultimately leading to cancer related genes silencing and/or activating. Another nuclear impact is observed on cell cycle regulation[174]. In line with this approach, it has been recently evidenced that the guardian of the genome p53, for example, was directly impacted by calcium signal and vice-versa[15, 175-177]. Hoth refers to those genes, which are impacted by calcium influx as the “calcium genes”[178]. An additional nuclear consequence is the change in the nuclear synthesis of some specific proteins. The goal here is now to increase extracellular calcium uptake, in order to reverse the too low concentration of intracellular calcium. This is probably the basic event underpinning the quasi-constant *over-expression* of specific elements of the calcium signaling toolkit as elicited by most of the experiments reviewed in this paper. In the same line, while overexpressing proteins of

the signaling toolkit dedicated to calcium uptake, the cell downregulates those limiting it, such as high calcium sensing molecules. This in turn, leads to a runaway of the whole cellular machinery, especially the calcium dependent mechanisms. The list is long and includes amongst others, evasion to apoptosis, neo-formation of actives peptides such as growth factors, PTHrP (in relation to the local increase in calcium in the extracellular milieu), or furin, all of them being potent contributors to metastasis[179] as well as cell differentiation [180]. The uncoupling of the to be cancerous cell might account for the loss of response to growth control signals, as well as for tissue disorganization and destruction such as inter cellular junctions (tight and gap junctions), commonly described as cancer cells distinctive. The extracellular accumulation of calcium ultimately contributes to microcalcification. The mechanism is firstly aimed to be protective. In order to counteract the hyper chemical reactivity of the ion in the extracellular milieu, some matrix elements are released to chelate calcium by forming crystals such as oxalate calcium and hydroxyapatite. The later is well known to be the most stable form of calcium in solution [181]. Accumulation of calcium also contributes to changes in membranar potential, thus increasing the motility of the cell and its potential to metastasize. The *increase phase* can be linked to the *calcium influx or calcium signal pathways of cancer* and the *habituation phase* to *the store dependent cancer pathways* as discussed above. Thus, beyond the apparent chaos and complexity of the cancer phenomenology, there is a logic: the survival of the cell to chronic aggression, through adaptative and corrective mechanisms, which unexpectedly lead it on the way to immortality through evading life control systems. This new angle of approaching the disease becomes only apparent when we focus on calcium, the signal of life and death. An interesting review released in 2010 by Parkash and Asotra lists key events in the cancer molecular phenomenology in which calcium wave intervenes[182], these are recapitulated on Fig. 9. We also provide an overview of the calcium and cancer pathways on Fig. 10.

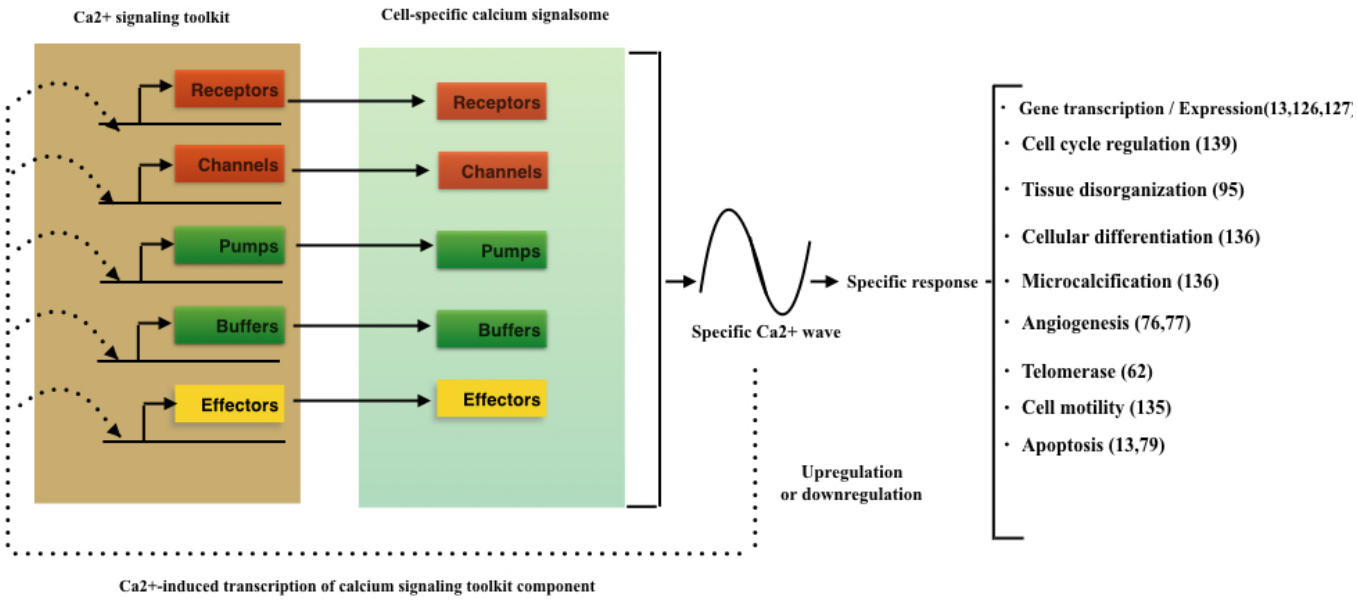

Figure 6: Calcium wave and cancer molecular phenomenology

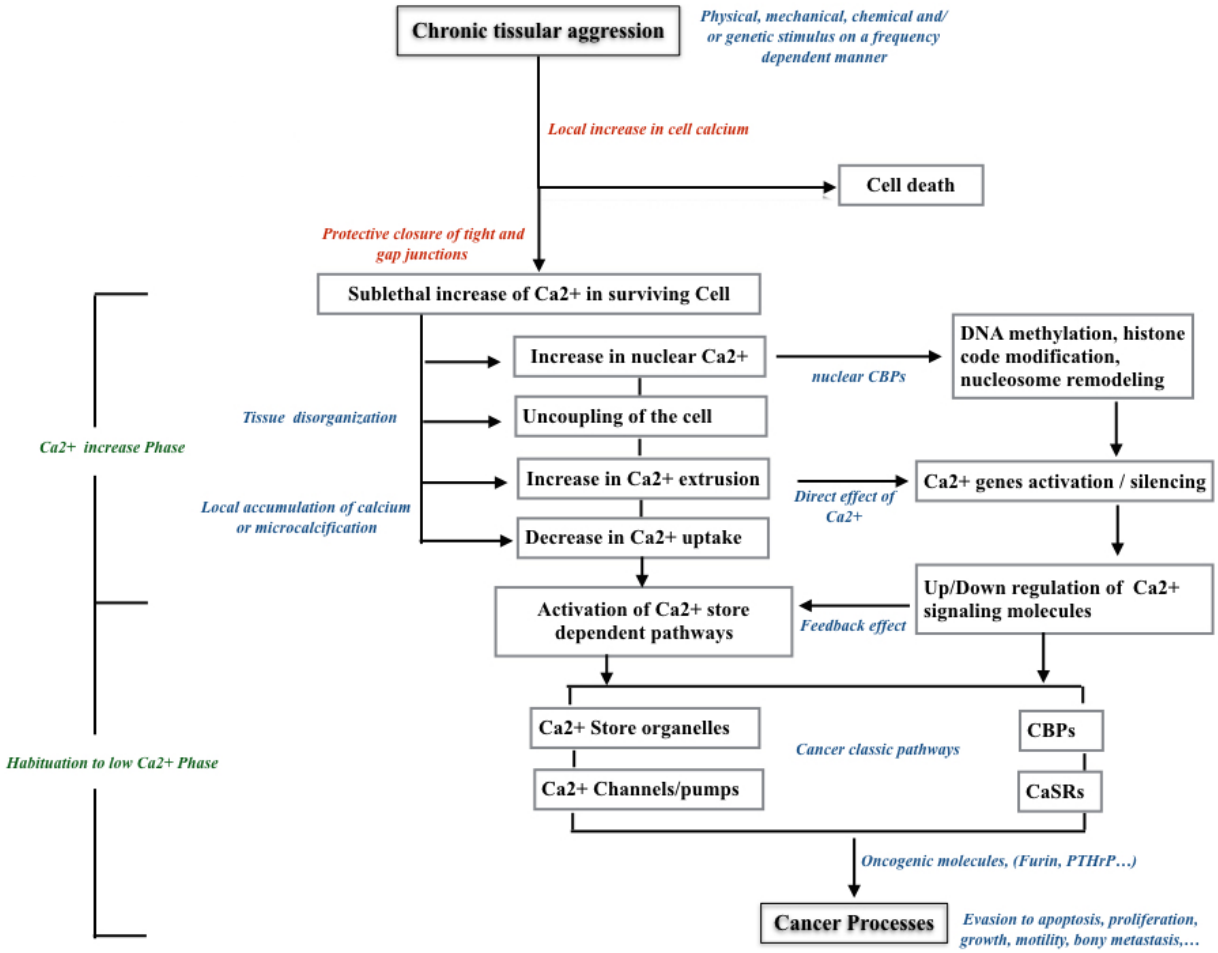

Figure 7: An overview of the Calcium Influx dependent Pathway

5. Implications and consequences of the calcium theory of carcinogenesis

5.1. Cancer diagnosis, treatment and prognosis from the calcium perspective

The coherency of a calcium based theory of tumor initiation, promotion and progression could be further enhanced by the practical implications it raises, in terms of the development of new clinical and therapeutically tools, or a renewed significance for some cancer hallmarks. Firstly, we mentioned *microcalcification* as a result of impaired calcium trafficking in tumorigenesis. Converging studies support its diagnostic and prognostic role in common cancers. Probably the most studied neoplasms associated with microcalcification are breast malignancies[183] . Two types of microcalcifications have been identified in breast lesions: Type I calcification or calcium oxalate versus Type II, which is essentially made of hydroxyapatite crystals. It is accepted that type I is mostly associated with benign lesions, whereas type II is correlated to malignant tumors [183]. In the recent years attempts have been made to predict the malignant nature and /or the prognosis of microcalcification in breast tumor based essentially on radiographic criteria. Predictive parameters such as their pattern (linear or aborescent), their number (>15 per cm2) or increased density have been suggested[184, 185]. A similarly attempt is under investigation with other neoplasms such as in thyroid[186], prostate[187] and testicular cancers [188]. Another potent diagnosis and prognosis feature deriving from the calcium perspective is the potential to develop new cancer biomarkers. As seen above, the expression of some specific proteins of the calcium signaling toolkit is significantly altered in different types of cancer. As mentioned in section 2, Chromogranin A for example is more specific than the classic PSA in diagnosing the prostatic neoplasms[130] . A recent work conducted by Wang and co-workers, suggested the S100P protein of the S100 family as a novel prognostic marker for colorectal cancer[189]. The authors compared S100P and mRNA in a series of no less than 96 samples of colorectal cancers versus control tissues and concluded to a significantly different overexpression of the target molecules. Another element of the calcium signaling toolkit fueling the calcium perspective on cancer is represented by the Calcium Sensing Receptors (CaSRs) of which mention has been made in the former sections of this paper (Section 2). The CaSRs play an important role in cancer and can serve as biomarkers of several type of cancer; the colon cancer in particular has been extensively researched [103]. Their interest as frontline sensors of local variations in calcium concentration could be critical in early diagnosis of the disease. We mentioned earlier that they were downregulated in colorectal cancers and in theory this model could be extended to other

tissues. The interest of the calcium approach in cancer is also therapeutic. The work of Mishra mentioned earlier referring to S100A4 posited that molecule as a potential target for cancer therapy. The key idea here, according to their work, is to inhibit/suppress S100A4 expression. Whenever that resulted was achieved in experimental animal models, a significant reduction of metastasis was observed. The authors report the development of S100A4 inhibitors capable of inhibiting the metastatic potential of prostate, pancreatic and skin cancer in vitro[190]. Also, Raynal's work cited earlier[169] shades a light on the possibility to reactivate silenced tumor suppressor genes using drugs that interfere with calcium influx at the cellular level. This is supported by the work of Montheith et al. conducted less than a decade ago in which the authors suggested calcium transport as a potential target for cancer treatment [191]. This argument has been further illustrated in their more recent publication[192]. Much more recently, works conducted on developing the SERCA pump inhibitors have also enriched the scope of developing new therapies based on the calcium hypothesis of cancer[193]. Seo and co-workers just published a work on the inhibitory role of *Curmin* on the SERCA pump [194]. Similarly, mention can also be made of calcium channel blockers [178, 192]. The general principle here is that a molecular therapy of cancer is envisageable using drugs aimed at reestablishing normal calcium signal in cancer cells, including calcium-based auto-destructive mechanisms such as apoptosis[192]. This is not quite new, as available data suggest interferences between some classic anti-cancer drugs, especially the metals such as *cisplatin*, *arsenic trioxide* and *trimethyltin*, and calcium signaling in cancer cells [195]. The advantage to target specifically calcium signal resides in the potential to selectively target cells with only impaired calcium trafficking. Finally, mention should be made of the longstanding work of Pace-Asciak and co-workers on Hepoxilins isomers, some unstable bio-metabolites of arachidonic acid. The authors have long ago established the role of the HX family in the molecular mechanisms underpinning chronic inflammation in eukaryotic cells. What is interesting here is that, the HX pathway provides additional support to the calcium theory of cancer. Hepoxilin A3 has been suggested to interfere with calcium transport and to inhibit the rise in free intracellular calcium [196, 197]. The mechanism serves as a basis for developing more stable antagonists namely, the Proprietary Bioactive Therapeutics (PBTs). In a more recent paper, Pace-Asciak reviewed the potential for the PBTs in some chronic diseases including cancer. Case controls experiments in both animal models or based on various human cancer cell lines demonstrated a dose-dependent effects of PBTs in reactivating apoptosis in cancer cells [198]. One certitude is that calcium signaling pathway is now in the agenda of cancer drug as commented by a grey literature article released online in 2015 under the title: *Calcium Signaling*

Pathway in Cancer Drug Pipeline Update 2015 (http://www.prnewswire.com/news-releases/calcium-signaling-pathway-in-cancer-drug-pipeline-update-2015-300107979.html). According to the article, close to 300 drugs targeting calcium signaling are currently under investigation.

5.2. Implication for public health: the role of dietary calcium

The calcium perspective on cancer raises some questions to which it is appropriate to provide some insights. One of these issues is the role of external factors and particularly of diet. The only source of body calcium being the dietary intake, could high nutritional calcium be considered as oncogenic? To date, most epidemiological studies conducted on the issue remain specific to some cancer types. Recently a general study has been published but the analysis included other cancer related diseases such as cardiovascular morbidity which renders a conclusion difficult to make for the overall cancer population[199]. Furthermore, as mentioned above, the versatility of calcium role in the cell machinery also translates in its role in cancer risk. For example, according to most observational studies, high dietary calcium, to a certain limit, is chemoprotective for colon and breast neoplasms, whereas for prostate cancer, it seems to be the opposite, although a consensus is yet to be reached[200, 201]. A key point transpiring from our review is that the carcinogenic increase in calcium concentration (*phase I*) is both a *local* and a *chronic* event[139]. By « *local* », we mean at the tissue level and, by « *chronic »,* we understand in a repetitive manner. Whilst high dietary intake directly affects the intestinal tissue, it might not necessarily translate into a local increase in the interior milieu tissues, given the relative efficiency of the broader homeostatic mechanisms in controlling calcium concentrations. The tissular uptake of calcium is under the dependence of a series of factors amongst which the most prominent is certainly calcitriol (1,25 OH-D_3), the active form of Vitamin D, although the compartmental concentrations of other minerals such as phosphate, zinc or magnesium, or some other organic molecules should also be considered. But the bioavailability of calcitriol is probably much more difficult to capture than generally accepted, as it is a direct function of external environmental factors including: race, age, sex, tissue type, and sunlight exposure as well as of some genetic parameters such as structural enzymes of the hydroxylase types. A common weakness in most of the observational studies on calcium and vitamin D roles in cancer is their failure to appropriately measure the later, due to their insufficient consideration of the sus-mentionned environmental factors. Stated otherwise, one measurement of vitamin D is more likely inappropriate to inform on the real Vitamin D status, hence on the calcium tissular uptake, if abstraction is made of sex, race, tissue

type, exposure to sunlight and other dietary habits. Thus, in researching the role of dietary calcium in a specific type of cancer, other life style factors should be also investigated and integrated to the precise calcium metabolism in the tissue in question. This points out the need for a multilevel approach, combining both epidemiological and molecular investigation to best capture the complexity of the issue. However, the fact of *eating* being a cultural habit, it fulfills *per se* the condition of *chronicity*, and should therefore be considered as a potential influencer of calcium role in cancer. The tissular uptake will determine the condition of *locality.* Here, a way forward is probably to be able to measure and predict the normal calcium uptake capacity of a given tissue type. Based on the colonocytes model, we suggest that the normal quantitative expression of CaSRs at the surface of the plasma membrane of a given cells serves as a marker for its calcium uptake capacity. As mentioned in earlier paragraphs, available data support that the most superficial layers of the intestine naturally express an elevated number of calcium sensors and this is believed to account for the protective effect of calcium in colorectal cancers [100]. The rationale here is that CaSRs are the frontline sensors of microenvironmental or local changes in free calcium concentration [98] and the more there are *naturally* expressed at the surface of a given cell, the more that cell is capable of appropriately managing high levels of calcium [202]. In other words, all external factors (race, sex, season…) being equal, it is more likely that high dietary calcium is potentially detrimental for tissues expressing a reduced number of CaSR at their surface and will be rather chemoprotective for tissues with high expression of CaSRs and vice-versa. This molecular versatility might have accounted for the conflicting findings in epidemiological studies. For an illustration of this *threshold effect hypothesis*, we will conducted an observational study using prostate cancer in Subsaharan Africa. This part of the world is well-known for its epidemiological transition, with a steady increase in cancer incidence, especially in prostate cancer[8]. It would be interesting to parallel that increase with changes in life style mainly eating habits with a focus on calcium. Based on the concepts developed here, we predict that a combination of risk factors with the potential to impact calcium cellular metabolism to be fueling the increase. The results of that study are discussed in the next chapter.

The rationale for conducting a critical review is twofold. The first aim is to provide a state-of-the-art on a science and, secondly to formulate new hypotheses on which future research could be based for the advancement of that field [203]. Over the past century, the *calcium and cancer literature* has grown significantly. But the deriving knowledge remained sparse and divided up into the different branches of cancer research. Our yearlong analysis of a significant portion of that literature, provides support that calcium cellular homeostasis is a key to a conceptual rethinking of carcinogenesis. Eukaryotic cells have the innate adaptative properties to maintain optimal concentrations of calcium by a constant intercompartimental trade of the ion. The deriving variations in calcium concentrations and the induced signal underpin universal life functions and control mechanisms over cell death, growth and division. We have hypothesized that tumor initiation, development and progression is supported by a chronic failure of the cell to ensure appropriate calcium concentrations in the different compartments. Thus we were able to correlate classic cancer pathways to those changes in calcium concentration. We classified them as *the calcium store dependent cancer pathways*. They share a common feature: calcium ion is involved as a second or sometimes a third messenger. A further analysis of the literature also allowed us to hypothesize that calcium could also function as a first messenger in cancer, in an *influx dependent manner*. We called this second mechanism the calcium *signaling cancer pathway* with the particularity of *calcium wave* being the principal vector. The conceptual coherency of our approach is validated by the promising ongoing cancer research for the development of new diagnosis, prognosis and treatment tools and options based on calcium signaling. But it also appeared that to the universality of calcium role in cancer, another important notion should be opposed: the concept of *versatility*. Calcium molecular action in cancer varies greatly, depending on criteria such as local tissular uptake, tissue type and the nature and the chronicity of the stimulus. It is certainly that molecular versatility that accounts for the conflicting findings in epidemiological investigations, as for calcium dietary intake and various cancer risks. However, if the teleology of cancer based on calcium signaling, shades new lights in understanding the disease, there are still some limitations and the way to a conceptual simplification of cancer remains long. Some concrete experiments integrating the various aspects outlined in this paper should remain to be performed, as well as a more multidisciplinary and comprehensive approach in researching the disease. It would be informative for our new theory to

correlate these finding with a study of prostate cancer and nutritional calcium intake in a population that have been relatively « free » from cancer for decades and now is facing increasing incidence. We hypothesize that such study should reveal changes in dietary calcium intake patterns.

Chapter 5: The Africa Prostate Cancer Study (APCS) : Design Aspect and Pilot Study (Study 2)

The second phase of our research was based on Research Question 2: *Is dietary calcium intake associated with the increase of prostate cancer incidence in Subsaharan Africa men? What is calcium intake pattern in this population and how does it compare with Western Africans ?* The objectives were as follow : *Study Objective 4:* To develop a standardized and culturally adapted instrument for measuring nutritional calcium in an African diet type and describe nutritional calcium intake patterns in a population of Subsaharan African males;*Study Objective 5:* To test the correlation between nutritional calcium intake and incident prostate cancer rates in a Subsaharan African male population;*Study Objective 6:* To compare nutritional calcium intake and prostate cancer risk between two African male populations, with a common genetic ancestry, but diverging by nutritional calcium intake patterns and other environmental exposures.

1. Study Method

Prospective cohort studies are indicated where case-control designs might be unethical, yet a rigorous methodological approach is needed to attest of the internal and external validities of the results. They also present with the advantage that the « cause » precedes the « outcome » investigated. Phase II of our research is a cohort study, conducted in Côte d'Ivoire. We developed a prospective cohort study preceded by a pilot and feasibility study.

1.1. Study Framework

The framework of the observational component of our investigation was borrowed from the Harvard-led *Pooling Project of Prospective Studies on Diet and Cancer*[62]. The *Pooling Project* is a worldwide research that assemble key prospective cohort studies aimed at investigating the association between nutrition and cancer risk. Cohorts from all continents are expected to be part of the project, however, no Africa-based study has been included to date, although African ancestry is a main risk factor to prostate cancer. Therefore, we designed Study N°2 after the methodological requirements for studies to be included in the *Pooling Project* [62]: (*i*) Population-wide prospective cohort design (*ii*) focus on diet and cancer and (*iii*) using a questionnaire that has been validated as study instrument. We called our study the African Prostate Cancer Study with the code name the -APCS-.

Côte d'Ivoire has been purposefully selected out of practicality, to host the study. Available data suggest that incidence rates in the country have been rapidly increasing in the recent years [204]. The pilot work was conducted with researchers from the Service d'Urologie de l'Université de Bouaké.

The PICO framework gives an overview of the study, but further details are provided in the next paragraphs: *Population:* retired civil servants selected in Côte d'Ivoire. *Intervention:* a population-wide prospective cohort follow up, free from prostate cancer at the onset of the study and in whom dietary information would be regularly collected. Prostate cancer is the principal O*utcome* and *Comparison* of incidence rates is made at two levels : *within* the study population high calcium intake participants versus low and, *between* the Subsaharan African population and African American males.

1.1.1-Cohort Description: Size, Age group, geographic distribution

The primary purpose of the APCS is to investigate the relationship between dietary calcium and prostate cancer risk in the Ivorian population. The French speaking nation of Côte d'Ivoire is 322 462 sq.km wide and comprises 14 geographic districts (Table 1). The District of Abidjan, the city capital, is the most prominent, hosting over 20% of the population. The country gained political Independence in 1960 and per World Bank data is now a leading and rapidly modernizing economy in West-Africa. The Ivorian population is rather young with 75% of the people being below the age of 40. It is estimated at 25 million with a 1.08 male-female ratio. Close to 45% of the population live in the rural areas. Men aged 50 y.o and over, (the pre-retirement age) represent approximately 5 % of the total male population as per the 2014 general census. Men's life expectancy is estimated at 53 [205]. Retired civil servants are members of different associations and represent a group of approximately 100 000 people. Published data on prostate cancer in the country are scarce especially when it comes to nutritional cancer epidemiology. The Ivorian national cancer registry is yet to be fully operational. A 2014 retrospective study covering a 24 year-period based on biopsy samples concluded that prostate cancer incidence age was 3 to 4 times higher between 65 and 75 y.o[204] . Adeloye and coworkers estimated its general incidence at 31.4 per 100 000 in the Ivorian population [27]. Finally, a non-published data in elderlies in the country suggested that mortality rate picks approximately 10 years after retirement. Based on these figures, we determined that : (1) a minimum 10 year-follow up of retirees between 55 and 75 years old, with data directly collected from participants on an annual basis would be the optimal design to test the study hypotheses; (2) a stratified sampling should be operated at

baseline in order to reflect age and spatial distribution of the at-risk population with 4 strata : (*i*) the districts, (*ii*) the area(urban or rural), (*iii*) the city of Abidjan and the (*iv*) different age groups. A higher number of participants, say 70-75%, should be before the age of 60; (3) Fleiss formula with Corrected Continuity (CC) for cohort size determination[206] (under the following assumptions : study two-sided confidence level : 95%; power : 80%; ratio of unexposed to exposed in sample : 1; percent of unexposed with outcome : 25; percent of exposed with outcome: 33, relative risk : 1.5 ; risk/prevalence ratio: 1.3; risk/prevalence difference 8.3), returned the value of 1294 as simple size, using the open source statistical software Openepi (Emory University, 2013). We estimated that this number would be a strict minimum, given that there are no strong previous data supporting our assumptions, and anticipating a 10% annual attrition rate in the cohort due to divergent endpoints (loss in follow up, death, refusal, displacement). The final sample size of the APCS should be at least 4 times the minimum computed. At baseline our study would therefore include 5 250 participants distributed as shown on Table 1, based on the demographics of each specific District as per the 2014 general Census mentioned earlier.

Table I: Distribution of the APCS Cohort.

Age groups	55-59		60-64		65-69		70-75		Total /District				
Districts	**Urban**		**Rural**		**Urban**		**Rural**		**Urban**	**Rural**	**Urban**	**Rural**	Total
Abidjan Metro (District 1*)	300		100		220		100		75	75	50	30	**950**
Montagnes (District 2)	150		50		100		50		30	20	20	20	**440**
Sassandra Marahoué (District 3)	150		50		120		20		30	20	20	10	**420**
Bas Sassandra (District 4)	100		30		100		30		30	10	10	10	**320**
GôhDjiboua (District 5)	100		30		100		20		20	20	10	10	**310**
Savanes (District 6)	100		30		100		20		20	10	10	10	**300**
Lagunes (District 7)	100		30		100		50		30	20	10	10	**350**
Vallée du Bandama (District 8)	100		30		100		50		30	20	10	10	**350**
Lac (District 9)	100		50		75		20		40	30	10	10	**335**
Comoé (District 10)	100		50		75		50		20	10	10	10	**325**
Zanzan (District 11)	100		50		75		50		20	10	5	5	**315**
Moroba (District 12)	100		50		50		30		20	5	5	5	**265**
Denguélé (District 13)	100		50		75		30		15	5	5	5	**285**
Yamoussoukro (District 14)	100		50		75		20		15	10	10	5	**285**
Total / Area	**1700**		**650**		**1365**		**540**		**395**	**265**	**185**	**150**	**5250**
Total /Age group	**2350**		**1905**		**660**		**335**		**5250**				

**District number were arbitrary assigned for practicality purposes.*

1.1.2-Cohort recruitment and follow up strategy

The recruitment will be performed in the local government offices of the Ivorian National Agency for Retired Civil Servants, when the retirees come in for their monthly retirement pension. They will be provided with an information kit comprising the consent letter for the study, and other key information about the research design and goals, and procedures on how the findings will be shared with the participants. Their contact information (mostly cell phone numbers), if they will to be part of the study, will be collected at that time. The research Team will then contact the participants directly for their consent and schedule a medical appointment for baseline screening and questionnaire administration. The District Hospitals will be the focal points in each District for medical data collection. Screening of prospective participants will consist in (*i*) blood total PSA, (*ii*) digital rectal examination (DRE) and recent urinary history and eventually (*iii*) a ultrasonography of the prostate gland. Whenever there is an ambiguity, a confirmation biopsy will be performed. To be included in the cohort, willing participants should have a PSA < 4 ng/l and normal DRE with no recent dysuria.

1.2.3-Statistical Analyses

Multivariate Cox regression models will be used to estimate hazard ratios (HRs) at a 95% confidence interval within intake quartiles in the cohort [207]. The analyses will be conducted at the desegregated level along the lines of the sampling strata. Stratification on year of follow-up will be employed to assert the influence of potential preclinical condition. Nested case-control will investigate specific dietary patterns and a sub-cohort of 1 500 participants will be randomly selected at baseline for studying the influence of both dietary and non-dietary factors in the cohorts. Case-cohort analyses will be conducted, in which the experience of the sub-cohort will be compared to the incident cases. Following the model of the Dutch Cohort, annual intra-individual dietary variations in the cohort will be asserted in a random subsample of 250 participants [208]. This process will also allow us to constantly update the study questionnaire as the study progresses. All the dietary analyses will be conducted after adjustment for confounding factors such as physical activity, socio-economic status, pre-existing condition.

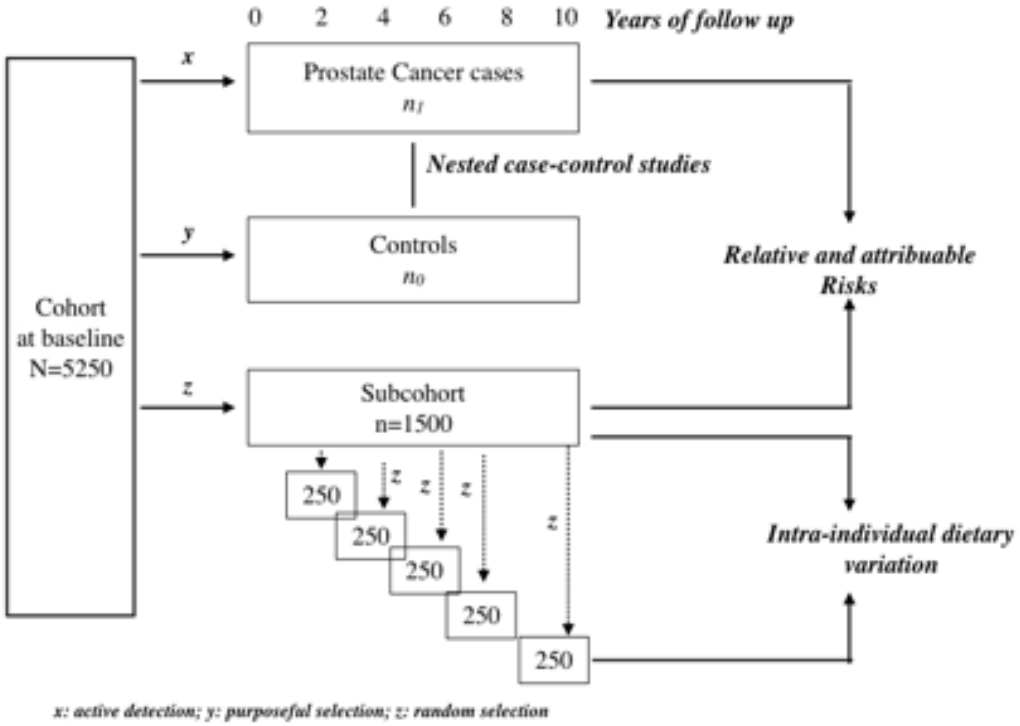

Figure 8: Design aspects of the African Prostate Cancer Study (APCS)

1.1.4-Study Instrument design

Calcium intake questionnaires abound in nutritional epidemiology thanks primarily to osteoporosis. Three questionnaires in particular presented with a certain interest for the APCS due to their popularity: Block calcium-vitamin D questionnaire[209], Fardelonne questionnaire which has the advantage to be well-known to French audiences[210] and the NIH-AARP questionnaire which is more general, but very detailed to capture wide intake range of calcium. They all had nonetheless a common weakness, being mainly based on a Western diet type[211]. The best option was then to use them along with other questionnaires, rather as guidelines for developing a new African diet-based calcium questionnaire. We will refer to our questionnaire as the APCS FFQ in the rest of this monograph. The general guidelines outlined by Cade et al.[212] for establishing robust FFQs in dietary assessments were followed in establishing the experimental APCS FFQ. As mentioned above, we benchmarked from different published models including: Block Calcium and Vitamin D[209], the NIH-AARP questionnaire [211], Fardelonne calcium questionnaire[40], the multiethnic cohort questionnaire [47] and the Adventist Health Studies, I & II[213, 214] among others. The final FFQ comprised two portions: a general information or non-dietary section and the dietary portion (Appendix 1). The non-dietary section collects participant's biometric and demographic background: age, height, weight, earlier profession, area of residence, year of retirement, socio-economic status, preexisting condition and medication, new medical condition (after the first year of follow up) dietary supplements, socio-economic status, physical activities, alcohol consumption and cigarette smoking. The section also records the results of the study medical visit: blood pressure, glycemia, vitals, total blood PSA and DRE. As for the food item selection, a list of common food categories in Côte d'Ivoire was obtained from the 2015 National Households Survey (ENV 2015)[215]. It was then compared with a previous work conducted by Dubresson back in 1989 [216]. To unsure comparison with similar work in other countries, we determined that the FFQ should also include foods that have been identified as potentially linked to prostate cancer risk in former studies, as reported by the CUP, the *Pooling Project* and other studies[217]. The final experimental questionnaire comprised 102 food items divided in 5 different categories: dairy products, soups, starch (rice, maize, plantain...) and tubercles (yam, manioc...), proteins (red meat, white meat, fish…), fruits and vegetables, legumes and fats and drinks. Finally, a blank space for *other foods* was left in case the applicant needed to add something from his diet that was not included. Some food are listed according to the local appellation, or are presented by dish. After the qualitative listing of food

items in the FFQ, the following step consisted in outlining a method for proper estimation of ingested food quantities and their calcium content. In a similar work conducted by Xu et al. the authors noted that a mass-based approach to evaluate ingested food quantities had the tendency to overestimate. They recommended instead a volume-based approach in which the respondents estimate their intake based on a photographic image of a standard portion size of the food item presented [218]. In this study the two approaches were combined. A reference portion for one person for each food item was ordered at the Bouaké University Restaurant under the supervision of a registered dietician. The composition in calcium in each food item was determined using different food tables according to the origins of the food item. For food originating in the traditional Ivorian gastronomy, we used the results of a survey (non-published) conducted by the Ivorian National Institute of Public Health. These data were compared with a food composition table for West Africa, published in 2011 by the Food and Agriculture Organization (FAO) [219]. Additionally, we used a food composition table published online by the Ministry of Health of a neighboring country, Burkina Faso, which share in commons many food habits with Côte d'Ivoire[220]. For food items originating from Western diet, such as dairy products or drinks, we used the French 2017 food composition table of the *Centre d'Information sur la Qualité des Aliments* (Ciqual 2017) [221]. For more international food items such as the pizzas, we used the Food Composition Table developed by SureQuest Inc. and published in the William's *Basic Nutrition and Diet Therapy*[222]. Frequency categories from the FFQ were converted to daily intake; thus, *never or rarely* was assigned a weight of 0; *1–3 servings/month* was assigned a weight of 0.067; *1 serving/week* was assigned 0.143; *2–4 servings/week* was assigned 0.429; *5–6 servings/week* was assigned 0.786; 1 serving/d was assigned 1; *2–3 servings/d* was assigned 2.5; *≥4 servings/d* was assigned 4.5 . Total daily calcium intake (mg/d) by the respondent was the sum of calcium contents in the different foods intake timed by the daily frequency [209].

1.2- Pilot Study design : Validation and reproducibility Study of the APCS Questionnaire

The validation study was conducted for 12 months to cover a full cycle of seasonal variations. We opted for a *Within-Subjects Design* (Fig.4). The experimental FFQ was first administered in February 2018 (FFQ1) then for a second time in January 2019 (FFQ2). Every 12 weeks, after FFQ1, the researchers administered at a random day, a 24h-Recall (24h-R) for a total of 4 measures (24h-R_j, j=1-4) per participant over the phone applying the usual five-step method for recalls [223]. The 4 recalls included at least one week-end and one week day. Stram et al.

established that the « ideal » number of days for dietary recording in validation studies will rarely require more than 4 or 5 diet records per subject[224]. The researchers in MEC conducted 3 different recalls[225]. Based on a review of former calcium questionnaires with quasi-similar designs [63, 64], we estimated that approximately a correlation coefficient of 0.60 between intake levels measured by the questionnaire and the average of the recalls could be expected. And at 5% significance level with 80% power, we would need approximately 110 participants to guarantee that the lower limit of the 95% CI of the correlation coefficient was at least 0.40. Conveniently, the team decided that the APCS FFQ should be tested in *District 8*, *Vallée du Bandama*, where the University of Bouaké Hospital and its Urologic unit are located. After ethic clearance from ERBs of both Universities, an agreement was signed with the local Association of Retired Persons. The recruitment was conducted in 2 phases in urban areas first, then the participants living in rural areas. A potential list of 120 participants was carefully selected to match the age groups of the final cohort. In that group, 5 individuals presented with an enlarged, hardened and irregular prostate gland at the DRE, associated with an elevated total PSA (>12 ng/ml) our cutpoint being at less than 4 ng/ml. They were classified as probable cases and were declared ineligible for the study. Another subgroup of 9 participants lived too far in the rural portions of the District and could not be reached for at least one of the recalls, trough the mobile phone system. They were regarded as *loss of follow up*. Finally, 4 contacted persons declined to be part of the study. Thus, the reproducibility and validation study was conducted with 102 participants (*n=102*), yielding to a study power of 74%. Furthermore, in their review of 227 FFQs over a 20-year period, Cade et al. recommended that validation studies sample size should be between 100 and 200 if dietary information are between 2 to 5 replicates (days) per subjects. For our study we had 6 measures.

1.2.1. Statistical analysis

Descriptive statistics were used for demographic information on study participants. Calcium intake distribution is presented per individual, as means, standard deviations, medians and interquartile ranges. A normality test (Shapiro-Wilks) was conducted on calcium intake (mg/d) distribution data from the different surveys and presented with a conclusive result ($p>0.05$). Pearson coefficient was therefore used for all correlational analyses. However, whenever normality was not consistent data were log-transformed to meet model assumptions.

Reproducibility (intra-method reliability)

A paired t-test was (p-value =0.05) performed on data from FFQ1 and FFQ2. The mean difference and 95% CI for calcium intake estimated from FFQ1 and FFQ2 and the intraclass correlation (ICC) were used to assess reproducibility. High ICC values, denoting low within-subject variability, reflect a high degree of reproducibility. According to the new guidelines provided by Koo and Li in 2016[226], ICC could be interpreted as follows:

- ICC < 0,50 : poor intraclass correlation
- 0.50 ≤ ICC< 0,75:moderate intraclass correlation
- 0.75 ≤ ICC < 0,90 : good intraclass correlation
- ICC ≥ 0.90: excellent intraclass correlation

1.2.2 Validity (inter-method reliability)

The mean difference and 95% CI for calcium intake estimated from the average of the 24h-Rs and the FFQs, de-attenuated Pearson correlations, attenuation factors were used to assess inter-method reliability. The de-attenuated correlation coefficient (r_d) formula is: $r_d = r_0(1 + \gamma/n)^{1/2}$ where γ is the ratio of the within- and between persons variances and n is the number of repeats (here n=4) and r_0 the observed correlation. De-attenuated correlations in the range of 0.4 to 0.7 were considered a priori to reflect inter-method reliability. The attenuation factors (λ) are represented by the slope of the regression line of the average of the recalls on the FFQ and provide a measure of the expected bias due to measurement errors. The real association between the disease and the nutrient intake, say RR_1 the relative risk, will be affected by the multiplicative scalar λ in such manner that $RR_1 = (RR_0)^{\lambda}$, where RR_0 is the observed association[211]. Attenuation factors closer to 1 indicate minimal bias, whereas lower values denote greater expected attenuation when estimating associations between dietary intake and health parameters. Inter-method agreement was visually plotted using Bland-Altman method with 95% limits of agreement[227]. A cross-classification analysis was also conducted to determine the power of the FFQ to discriminately classify the study participants into categories based on calcium intake levels, in agreement with the recalls [228] . For that analysis, the cut-points of the different quartiles of calcium intake were first determined using increments of 400 mg/day. In the Continuous Update Project (CUP), dose-response analyses supported that there was an increase of 1,05 (95%CI: 1,02-1,09) in RR for total prostate cancer per 400 mg/day increase in dietary calcium[49]. Then we considered the different thresholds of dietary calcium of the 11 studies included in the CUP report and conveniently selected those better matching with the intake profile of our testing group. Hence, we set the first quartile (Q1) at 600mg/day and the third (Q3)

at 1200 mg/day. Each participant was classified in the lowest quartile and in the highest by both methods using the cut-points. We then compared the proportion of subjects correctly classified and misclassified by the FFQ using the recalls as a reference. Cohen's kappa statistic was used to assess the agreement of classifying participants into similar quartiles of daily intake. Sensitivity, specificity, positive predictive value and negative predictive value of the questionnaire were evaluated as well. along with predictive values. Sensitivity was defined as the proportion of subjects with daily intake ranging in the lowest quartile according to the recalls who also fell in that same quartile according to the FFQ and specificity was the same scheme but for the highest quartile. R software, version 3.5.3 (The R Foundation for Statistical Computing) and Excel 2016 (MS Windows) were used for the analysis.

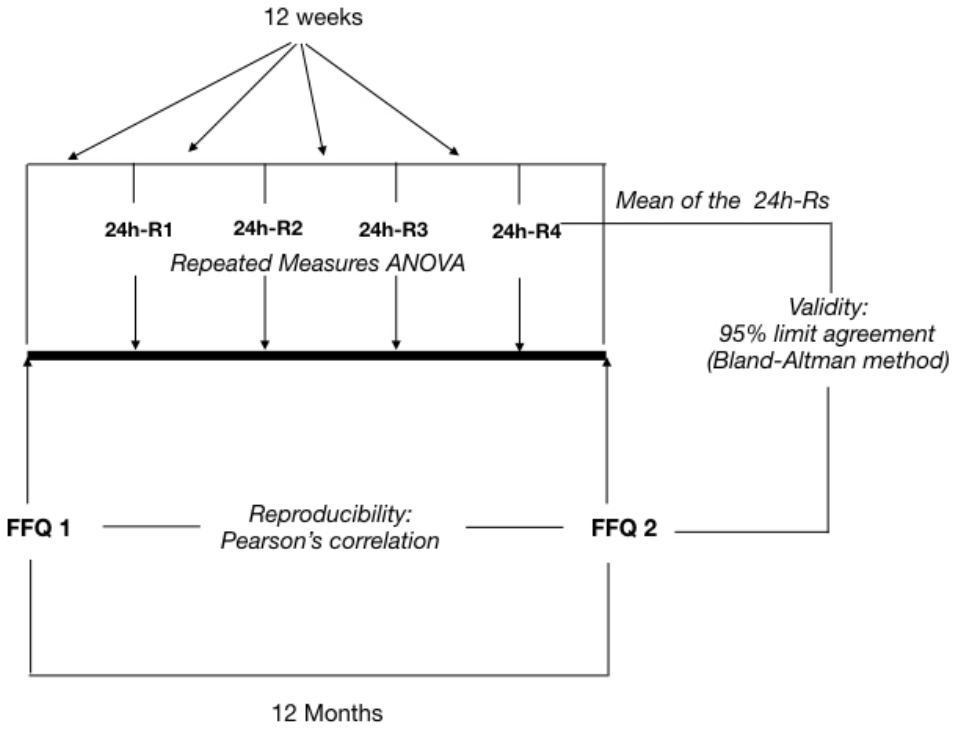

Figure 9: APCS Pilot Study Design

2. Results

Findings from the pilot study and the design aspects of the APCS are under review with the BMC related journal : Pilot and Feasibility Studies[2].

The APCS will be a nation-wide prospective cohort of 5 250 retired civil servants aged between 55 and 74. Case detection will consist of data directly collected from participants. A case-cohort approach will be applied, in which detailed follow up information of a random sub-cohort (*n=1500*) provides an estimate of the person-time experience of the cohort. Data from the sub-cohort will be analyzed with those of the incident cases, yielding exposure-specific incident ratios. The intra-individual variations in exposure, dietary intake of calcium, will be evaluated by annually repeated measurements (n=250) within the sub-cohort. As mentioned in Chapter 3, the Feasibility and Pilot Study (n=102) was conducted with the Urologic Unit of the Bouaké University in 2017 and 2018. The aim was to develop a standardized and culturally adapted instrument for measuring nutritional calcium in an African diet type and to describe nutritional calcium intake patterns in a population of Subsaharan African males (Study Objective N°3). It should also be noted that study questionnaire validation was an inclusion criteria for the APCS to be eligible to the international study conducted under *the Pooling Project of Prospective Studies of Nutrition and Cancer* [62].

*2.*1. Pilot Study Participants Characteristics

Mean age of the validation study group was 59,37 years (SD=3.92) and participants between 55-65 represented 80% of the sample. The group comprised mostly former school and university teachers (44%). In terms of life style smoking was not prevalent (10%) but regular alcohol consumption was high (63%) and physical activity inconsistent. Notable preexisting medical conditions included, HIV infection (7%), Hypertension (26%) and diabetes (14%). No specific diet, nor calcium supplements was reported, although certain respondent (14%) declared regular use of multivitamins. Digital rectal examination was within the normal limits for all the participants and mean PSA was 2.87 ng/ml (±0.8) (Table II).

[2] Pilot and Feasibility Studies - PAFS-D-19-00167

Table II: Descriptive characteristics of the validation study group (n= 102)

Age (Years)	*55-59*	*71 (69%)*
	60-64	*23 (22%)*
	65-69	*5(4%)*
	70-75	*3(2%)*
	Mean age	*58.07 (+/- 3.92)*
Marital status	*Married*	*86 (84.31%)*
	Non married	*16(15.69%)*
BMI (Kg/m2)	*underweight(<=18.5)*	*7 (4.9%)*
	Normal (18.5-25)	*66 (64.71%)*
	Over weight (25-30)	*21 (20.59%)*
	Obese (>30)	*8 (7.84%)*
Former Profession	*Teaching*	*45 (44.12%)*
	Military	*11 (10.78%)*
	Police	*8 (7.84%)*
	Administration	*15(14.76%)*
	Postal services	*4 (3.92%)*
	Health & medic.	*12 (11.76%)*
	Others	*7 (6.86%)*
Smoking	Yes	11 (10.78%)
	No (never)	76 (74.51%)
	Stopped < 10 y	15(14.76%)
Alcohol consumption	*Yes*	*65 (63.73%)*
	No (never)	*14 (13.73%)*
	Stopped < 10 years	*23 (22.55%)*
Physical Activities	*Regular (over once/week)*	*37 (36.27%)*
	Irregular (less once/week)	*29 (28.43%)*
	No	*36 (35.29%)*
Medical history	*HBP*	*28 (27.45%)*
	Diabetes	*16 (15.68%)*
	Arthritis	*45 (44.11%)*
	HIV infection	*4 (3.92%)*
	No	*9(8.82%)*
DRE	Within the normal limits	102 (100%)
PSA	<4ng/l	102 (100%)
Special diet	No	102 (100%)
Dietary supplements	*Calcium*	*0 (0%)*
	Vitamins	*15 (14.71%)*
	Herbal	*5(4%)*
	None	*82(80.33%)*

2.2. Dietary patterns and Calcium intake in the test-study group

A total number of 6 surveys were conducted over a period of 12 months . Mean calcium intake was 1023.41 mg/day (±297.81 mg/day) and 1076.16 mg/day (±321,74 mg/day) for the questionnaires (respectively FFQ1 and FFQ2). As for the recalls (24h-Rj, j=1-4), it was 1035.60 mg/day (±322.84mg/day) for R1, 1044.22 mg/day (±293.79 mg/day) for R2, 1011.21 mg/day (±287.22 mg/day) for R3, and 1072.37 mg/day (±319.33 mg/day) for R4. The total average of all 6 surveys (*n=408*) was 1043.83 mg/day (±307.13 mg/day). The different dispersions aspects of intake distribution per survey are represented with box plots on Fig.11.

- ***Dairy products***

Consumption of dairy products was low in the study population: intake was less than once a week (< 1/w) for 70% of the participants (*n= 102*). For the remaining 30%, intake frequency was between 1-2/w. For this latter subgroup, whole milk was the most common dairy product (43%), followed by coffee milk, locally called « *café au lait »,* and taken at breakfast time (41%), and yoghurt (39%). Intake levels of dairy products varied between 1 glass (67%) to 2 glasses (33%).

- ***Main Sources of Calcium***

Based on the different food composition tables used for this research, various sources of calcium were, mainly based on dried plant foods and fish (*Table III*).

- ***Red meat***

A similar pattern to dairy products intake was observed in red meat consumption. Close to 65% of total participants ate red meat less <1/w and the frequency was between 1-2/w in 25% of them. Cooked beef in a soup was the most frequent form of preparation (59% of total participants), followed by grilled beef, sold on the streets in small portions of approximately 15 mg each and locally called « *choukouya »* (41%). Wild game consumption was mentioned by 30% of total participants, with a frequency varying from 1-2/w to 3-4/w.

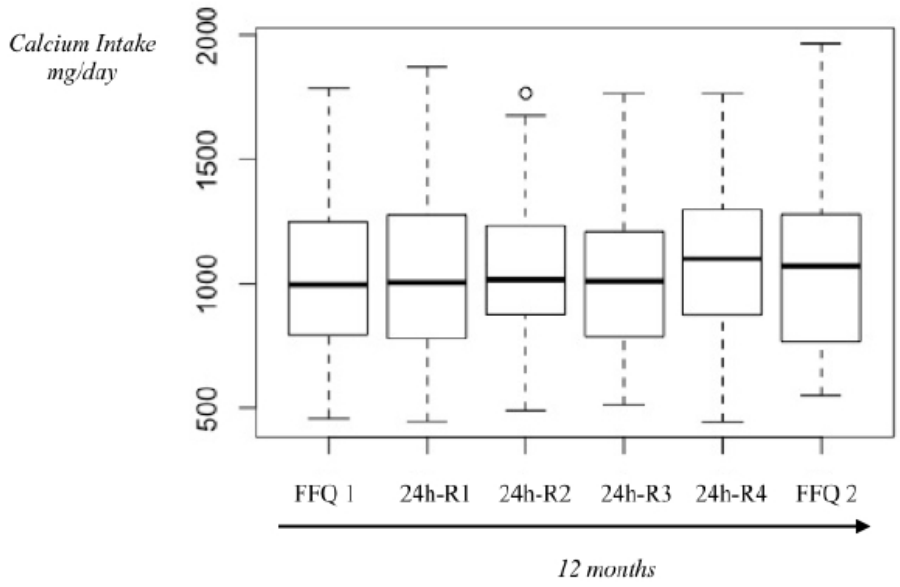

Figure 10 : *Calcium intake distribution in the study during the 12-month survey.*

Table III : Main sources of calcium in the study group

Food Item	Calcium content (mg /100 g)	Food Composition Table
Dried and fresh Okra *(Abelmoschus esculentis and Abelmoschus caillei)*	300-600	FAO, MOH BF*, Nix
Dried baobab leaves (*Andansonia digitata*)	2266	MOH BF
Dried fish with bones	1840	MOH BF
Dried small fish	1018	MOH BF
Dried Tilapia	2406	MOH BF
Sardines	400	Ciqual 2017
Whole milk (Powdered)	960	Ciqual 2017
Whole sweetened condensed milk	273	Ciqual 2017

* *Ministry of Health, Burkina Faso*

- ***Fish***

Fish was the main source of protein in the study population. It was present in the diet in two main forms, *dried* or *smoked,* although fresh fish is mentioned. A vast majority of the participants (83%) mentioned *smoked fish* in their diet at least 1-2/w up to 7/w. Similarly, 1 participant in 3 mentioned *dried fish* in their daily diet (7/w). Only 26% of the participants mentioned *refrigerated fish* in their weekly diet. The different fish mentioned by the participants were: *tilapia*, *catfish*, and *carp* for fresh water fish, and *herring*, *sardines* and *tuna* for salt water fish.

- ***Ivorian soups and starch***

An Ivorian traditional soup, made essentially with okra (*Abelmoschus esculentis* and *caillei)* eaten with steamed rice, with pounded yam, with manioc, or with pounded plantain mixed with manioc, was the most frequent dish in the study population. It is cooked in two ways: either with a powder of *dried okra,* locally called « *sauce gombo sec »*, or « *sauce djoumblé »*, or with *fresh okra,* called « *sauce gombo frais* » or « *sauce kopè* ». Close to 50% of the study group ate the « *sauce gombo sec* » 1-2/w, and for 30% of them the frequency was 3-4/w. For the « *sauce gombo frais »,* the fresh okra soup, intake frequency was as high: 1-2/w in 40%, and 3-4/w in 20%. The complete list of the different soups mentioned by the participants is provided as an appendix to this monograph.

2.3. APCS Questionnaire Reproducibility

On average FFQ2 recorded higher calcium intake levels than FFQ1. The mean difference of the intakes was 52.76 mg/day (±253.23mg/day) ($p<0.05$). Intake range was also wider for FQQ2. The interclass correlation coefficient (ICC) between the two surveys was 0,684 ($p < 0.0001$) with a 95%-CI varying from 0.566 to 0.775. Critical values of FFQ1 and FFQ2 are reported on Table IV.

Table IV: Comparative data between FFQ1 and FFQ2

	FFQ1	**FFQ2**
Survey date	Fev. 2018	Jan. 2019
Mean calcium intake (mg/day)	1023.40	1076.16
SD (mg/day)	297.80	321.74
95% CI of the mean (mg/day)	[920.52-1039.47]	[968.07-1092.23]
95% CI of the sample (log scale)	[480.18-1992.70]	[507.31-2084.82]
Minimum intake (mg/day)	459.19	559.43
Maximum intake (mg/day)	1787.23	1968.03
First quartile limit (mg/day)	798.58	765.21
Median intake (mg/day)	997.35	1071.12
Third quartile limit (mg/day)	1244.36	1278.48
Interquartile range (IQR)	445.77	513.26
Mean difference FFQ1-FFQ2 (mg/day)	-52.76	
SD of the mean difference	253.23	
ICC 95% (log scale)	0,657 ($p<0.0001$)	

$t = -2.2179$, $df = 101$, p-value = 0.0288 ($p<0.05$) (paired-test on log-transformed values), 95%CI

2.4. Inter-method reliability

There was a mean difference (*d)* Calcium intake $_{FFQ1}$ - Calcium intake $_{Recalls}$ of -17.45 (±220,75) mg/day (95% CI: [-60.81 to 25.92] but that difference was not statically significant (p=0.4266). There was a strong correlation between the two methods with a de-attenuated Pearson's coefficient at 0.701. Bland-Altman scatters exhibited a good overall between-method agreement (Fig.11). The 95% limits of agreement was comprised between -450mg/day and 415 mg/day (95%CI: [-525.24-490.34]). However, the between methods agreement was stronger when calcium intake was higher and less fair at lower levels. Furthermore, close to 10% of the scatters were beyond or very close to the edges of the confidence limits and the questionnaire also exhibited a slight tendency to overestimate smaller intake levels in the group and to underestimate higher intake levels (Fig.12). The attenuation factors coefficient (λ) of the questionnaire relatively to the recalls estimated at 0.636 (Fig.13).

Based on the cross-classification by the two methods, a performance table of FFQ1 in classifying the study participants was obtained (Table V). It derives from the performance table that the overall agreement between the questionnaire and the recall in classifying the testing study participants under the threshold of 600 mg/day posited as « *the lowest quartile of calcium intake* », was 42%. Cohen's weighted Kappa coefficient was 0.398 and sensitivity was estimated at 29%. In that same quartile, the positive predictive value (PPV) of the instrument was 36% and the negative predictive value at 83%. As for classifying the participants in the « *highest quartile of intake » (>1200 m/day),* the agreement with the recall was 38% and Cohen's coefficient: 0.453. Specificity in this interval was 61% with a PPV of 67% versus a NPV of 82%. Given the tendency of the questionnaire to inflate lower intake levels and to deflate the higher ones and the regression line zero intercept being around 900 mg/day (Fig.4), the performance of the questionnaire was reevaluated using 900 mg/day as an « *adjusted»* highest intake quartile. For that new threshold, specificity of the questionnaire was 85% and Cohen's kappa was 0.637 (Table VI).

Table V: Comparison between FFQ1 and the average of the Recalls (R)

	FFQ1	Recalls
Mean calcium intake (mg/day)	1023.40	1040.85
SD (mg/day)	277.80	215,35
95% CI of the mean (mg/day)	[920.52-1039.47]	[1987.77-1097.92]
95% CI of the sample	[338.39-1708.43]	[419.27-1662.44]
Minimum intake (mg/day)	459.19	527.64
Maximum intake (mg/day)	1787.23	1616,07
First quartile limit (mg/day)	798.58	858.16
Median intake (mg/day)	997.35	1065.17
Third quartile limit (mg/day)	1244.36	1245.93
Interquartile range (IQR)	445.77	387.76
De-attenuation coefficient	1.002	
Attenuation factors coefficient (λ)	0.639	
Observed Pearson Coefficient(r_0)	0.701	
De-attenuated Pearson Correlation (r_d)	0.701	
Mean Difference (*d*) (mg/day)	-17.45 (±220,75)	
95%CI of *d*	[-60.81-25.92]	
95% limits of agreement	[-450.13-415.23]	
95% CI of the limits of agreement	[-525.24-490.34]	

t = 0.79819, df = 101, p-value = 0.4266 (paired-test), (p>0,05) 95%CI

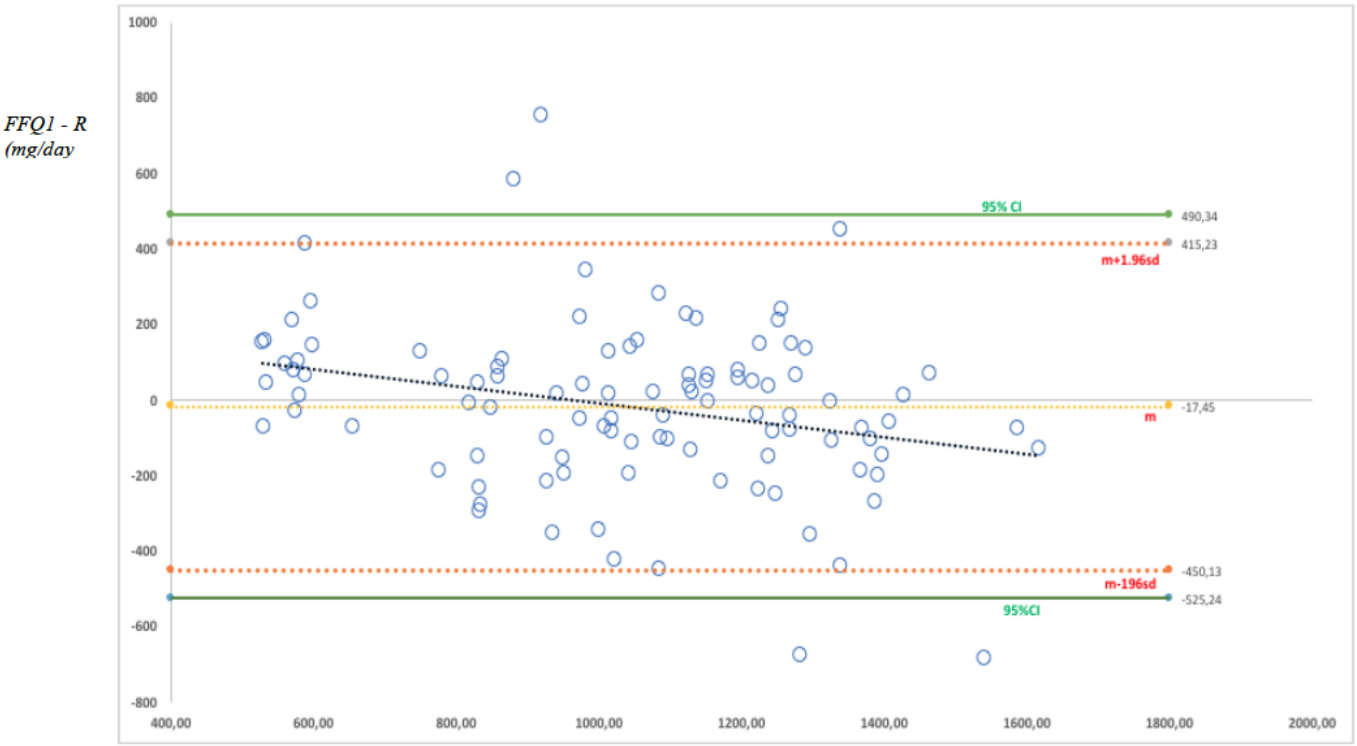

Figure 11: Bland-Altman Plots comparing FFQ1 to the recalls

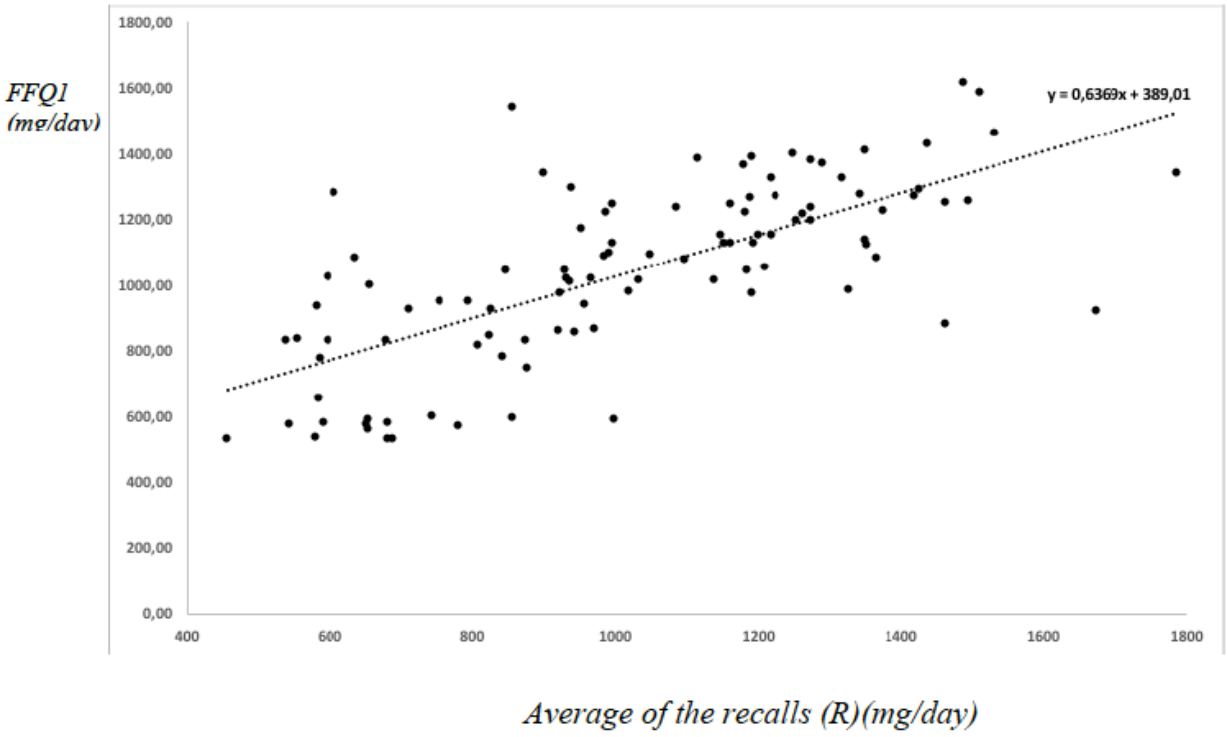

Figure 12 : Attenuation Coefficient of the APCS FFQ

<u>Table VI</u>: Performance of FFQ1 in classifying study participants relatively to the 24h-Rs

	Intake quartile			
	Lowest : (<600 mg/day)	Highest (> 1200 mg/day)	*Adjusted Highest* (>900 mg/day)	
a	True Positive*	4	20	62
b	False Positive	7	11	5
c	False Negative	10	13	11
d	True Negative	81	58	24
	Total	102	102	102
		$\varkappa$=0.398	$\varkappa$=0.453	$\varkappa$=0.637

** positive here means that the participants belongs to the right quartile as per the 24h-Rs classification.*

2.5. Analysis of the results of the Pilot Study

Unsurprisingly, calcium FFQs for dietary surveys in African men are scarce in the nutritional epidemiology literature, as the science is just now burgeoning in the emerging nations[229-231]. But even in more affluent countries, adult and aged women have the lion share of the nutritional calcium research, followed by adolescents and children, men and older men particularly are relatively underserved [232, 233]. We developed a new instrument, the APCS FFQ, based on food items typical to the Ivorian diet to investigate diet in men over 55. The testing study included a group of 102 volunteers randomly selected to which we applied a test-retest within-subject design. Such a design is commonly used in the FFQ validation literature[228, 234] and presents with the advantages to robustly evaluate both reproducibility and validity[235]. Mean age of the testing group was 58,07 years (+/- 3,92), mostly composed of former professionals from teaching, armed service, health and administration officials. The group had no particular dietary background, obesity was less than 10%, but medical history outlined high prevalence of arthritis (43%) arterial hypertension (26%) and diabetes (17%), confirming the rise of chronic diseases and other metabolic syndromes in the African population[236]. Diabetic patients in the study group did not report any specific diet restriction neither. The overall mean daily intake in the group was 1043.83 mg/day (±307.13 mg/day). This number is higher than generally expected in African populations [84, 85] and is also higher than those reported in their African American counterparts. In the Multiethnic Cohort (MEC)[17] average intake was 771 mg/day, a similar number to Glovioell's findings in the Californian Cohort: 881 mg/d[67] and Batai's in the Chicago study: 596 mg/d[54]. A recent African study, however, did find an intake average close to ours, 1241 mg/d but the participants were from the Northern part of the Continent[237]. Diet specificities might account for the variance. In effect, different determinants influence food choice and nutritional habits[238]. Older generations of Equatorial Africans have enjoyed a long tradition of a diet shaped by seasonality and biodiversity. In-season foods are generally cooked fresh from the farm, and out-of-season-foods are first sun-dried, smoked or grilled. This general pattern seems to influence the calcium content in their diet as confirmed by the present research. Dried plant-foods are generally higher in calcium compared to their raw form. The dried leaves of baobad (*Andansonia digitata*), used in the « *sauce lômi* » for example, could contain up to 2266 mg of calcium per 100g . As for the "*sauce gombo frais*" quasi constant in the diet of the pilot study group, in 2013, Kouassi et al. conducted a work on the two species of okra grown in Côte

d'Ivoire: *Abelmoschus esculentis* and *Abelmoschus caillei.* The research found that calcium content in these two species was high, especially when they were dried, both had close to 700 mg of calcium per 100g[239] . Without prejudging their bioavailability, this is higher than the calcium content in 3 to 4 glasses of whole milk. The low consumption of dairy products in the study group can be attributed to several factors. Cattle farming is not highly prevalent in equatorial Africa, imported dairy products are generally expensive and require appropriate ways of conservation and the study group belongs to a *pre-globalization* generation, before the country was exposed to international trade. The main source of animal protein was dried fish, although both red and white meat were present. Fish are dried generally whole with their bones, and for example 100g of dried tilapia could contain up to 2406 mg of calcium. Added to the generally long cooking time, it appears that traditional African soups can be very high in calcium. In total it appears that the typical Ivorian diet consists to a great extent of sun-dried leafy vegetables and vegetables such as okra (*Abelmoschus esculentus*)[239] or baobab (*Andansonia digitata*) and sun-dried small bony fish of the sardines type. It is well-established that such a diet is much richer in minerals, including calcium and other oligo elements with even higher bioavailability when compared to diets essentially based on animal products[240] . This once again emphasizes the need for FFQs to be cultural adapted [241]. Reproducibility of the questionnaire was evaluated by computing the intraclass correlation (ICC) between the test and re-test surveys conducted 12 months apart. The intraclass correlation coefficient ICC was 0.657 ($p<0.001$) which ensured moderate reproducibility by Koo and Li standards[226]. But for nutritional surveys a threshold of 0.64 is generally used and an ICC between 0.60 and 0.74 is considered reasonable[212, 242] . Our number ranges between those of Miller and El Kinany in similar designs, the authors reported ICC values at 0.6 and 0.78 respectively [237]. The determinants of ICC in nutritional research are debated and depends on several factors pertaining both to the questionnaire, to the target population and to the model used in the computation[230]. We imputed the good reproducibility of the APCS FFQ to the highly extensive list of the food items (102) which captured seasonal variations in diet, to the professional background of the study group, composed of people coming from highly intellectual professions and to the study design that maintained constant interaction with the participants. In a simplistic approach, validity can be defined as the capability of the questionnaire to capture values that are within the limits of « true intake » as measured by a reference method providing measurement errors are not significant[230]. The de-attenuated correlation between the questionnaire and the average of the recalls was 0.701 ($p<0.001$) which shows that intake values captured by the APCS FFQ are strongly correlated to

those of the recalls. Our coefficient is relatively high compared to those generally reported in similar studies: Magkos reported an attenuated coefficient of 0.639[232] , El Kinany, 0.55 [237], Osowski, 0.31[243] and Corrente 0.5[244]. A lower within-person variance in our study group could possibly explain our number. We postulate that the perceptive fear associated with prostate cancer, combined with the lack of access to cancer treatment care in our setting, in a group already afflicted with chronic diseases, might have provoked the respondents to « religiously adhere » to their diet once they were declared eligible for the study. The health belief model has been extensively utilized in former studies to explain results of individuals and groups in relation to health behaviors[245]. Bland-Altman plots confirmed the good agreements between the new questionnaire and the recalls but some limitations should be mentioned. The range of the 95% CI of the limits of agreement is important : from almost -525 mg/day to 490 mg/day. In other words the questionnaire could return intake levels that are as low as 500 mg/day and as high as close to 500 mg/day compared to « true values » giving a span of 1000mg/day. Such a span limits the quantitative accuracy of our questionnaire to measure « true » calcium intake at the individual level, which was not the intent in designing such instruments[230]. The interest of FFQs rests in their capability to discriminately classify individuals into different levels of intake in comparison with a certain cut-point[232]. While the APCS FFQ recorded a low sensitivity (29%) with a low agreement with the recalls for classifying participants in the lowest intake quartiles, the instrument recorded better performances in identifying subjects in the highest quartiles. The specificity was then 61% with a kappa coefficient yielding to a moderate agreement (0.45) with the recalls. These results are opposite to those reported by Miller and Clover in the Australian study [228]. The author recorded a higher sensitivity at 86% and a relatively low specificity at 57% for their 35-item FFQ and 82% and 46% for their 15-item questionnaire. Magkos et al. reported a 82.5% sensitivity[232] and general records in the literature report a lower misclassification rate of the range of 1.2% to 3.4%[246, 247]. However, it should be mentioned that most of the studies cited were more geared toward osteoporosis in which high sensitivity questionnaires were required for validity whereas in prostate cancer it is the increase in calcium intake that is the target, as per the CUP report among others[48, 53, 217]. In other words, specificity in the context of our study is prior to sensitivity, low calcium intake being even protective against prostate cancer according to some authors[55]. The classification performances of the APCS FFQ should also be analyzed in light of its tendency to overestimate low intake values and under estimate high intakes (Fig.6). That phenomenon is well-known for FFQs and has been described earlier as the « *flat-slope syndrome* » [232]. The zero intercept of the

regression line of the Bland Altman plots is the threshold point of the phenomenon: below that point intake levels are generally inflated and beyond they are deflated. That point in our study is at around 900 mg/day (Fig.6) which is also the adjacent quartile to the highest. The classification performances of the instrument increases dramatically when that value is used as the cut-point, specificity becomes 85% and Cohen's kappa coefficient new value is 0.635. This confirms the good ability of the questionnaire to classify subjects in the highest quartile. Those reported by the instruments as low intakes may have in reality their « true» intake even lower showing a potential interest for using the APCS FFQ in other calcium-related chronic diseases such as osteoporosis. The attenuation factors (λ=0.639) of the questionnaire relative to « true » relative risk is also worth of few words. The coefficient λ is closer to the unit which means that minimal attenuation of RR will occur when the instrument is used in survey settings. Ollberding et al report a lower λ in African Americans in a multiethnic cohort design (0.2) but it should be noted that the age group of their study population was different than ours[248].

Partial Conclusion 2

From a methodological standpoint, a large-scale prospective cohort study appears feasible in Côte d'Ivoire under the requirements of the *Pooling Project.* The African Prostate Cancer Study (APCS) as designed, could capture the relationships between dietary calcium intake and prostate cancer. The study instrument, the APCS FFQ, meets the general standards of the art and has demonstrated capability and consistency in capturing calcium intake in a group of older men over a period of 12 months. The instrument is reproducible and shows a good agreement with a 24 h recall although it has the tendency to overestimate lower intakes and underestimate higher ones. The typical Ivorian diet is rich in calcium although poor in dairy products. Calcium is mostly provided through sun-dried foods including, dried vegetables and and fish. Such intake pattern diverge greatly from the diet of the Diaspora Africans especially in Western countries where dairy in the main source of calcium. The APCS will surely bring new insights in prostate cancer epidemiology, fas we study further this population in nutritional transition. However, that initial qualitative and quantitative difference between traditional African diet and western diet is noteworthy in our exploration of prostate cancer disproportionate burden. Given the essential difference in intake patterns, are prostate cell lines in Africans interacting differently with calcium when compared to prostate cell line in caucasians? In the next chapters we discuss this question using a proteomic research design.

Chapter 6 : Proteomics (Study 3)

The last study of our research (Study 3) is a proteomics research and addresses the following Research Question : *Is there a differentiated cellular response when prostate cancer cell lines isolated from patients with different ethnoracial backgrounds are exposed to a synthetic calcium binding peptide ? What protein(s) or group of protein are then deregulated ?* Hence the following study questions are addressed : *Study Objective 5:* To synthesize a highly calcium specific peptide that would influence calcium metabolism in prostate cancer cell lines; *Study Objective 6:* To evaluate and compare the responses of prostate cancer cell lines of African origin to cell lines of Caucasian origin when both are exposed to a high affinity calcium binding peptide; *Study Objective 7:*To identify the dysregulated protein (s) when prostate cells from African cell lines and from Caucasian cell lines are exposed to a highly specific calcium binding peptide.

1. Study Method:

The proteomic portion of our investigation has two main objectives: (*i*) to explore the potential disparities at the cellular level when different prostate cancer cell lines are exposed to a synthetic molecule that influence calcium metabolism, (*ii*) to provide empirical support to the epidemiological and reviews portions of the research. The general method of this study is to synthesize a highly specific calcium binding peptide (CBP) and observe its effects *in vitro* on the malignant behavior of two cell lines : the *LNCAP* cell lines deriving from a caucasian origin and the *E006-AA-ht* cell line African American in origin. The ultimate step consist in identifying the protein profiles that are dysregulated to explain the cancerous cellular behavioral response. LNCAP , isolation and culture methods have been already extensively described in the literature[249], here we focus on the newly developed E006AA-ht cell line. The implications of this portion in terms of practical approach to medical treatment options of Prostate cancer are very important. In effect, If there is a difference in response then medical treatment options for PCa should consider ethnoracial adjustments.

1.1.- The E006AA-hT prostate cancer cell line

In 2004, Koochekpour and coworkers established the first PCa cell lines deriving from a primary tumor in an African-American PCa patient[250]. the cells were obtained from a 50-year-old

patient, after a radical retropubic prostatectomy for treatment of clinically-localized tumor. The E006AA, as it has been named, with the « E » for *epithelial,* were obtained along with another line of stromal cells named S006AA. E006AA cell lines express the androgen receptor (AR) and are Androgen sensitive with the unique particularity to attain maximal growth for relatively small concentrations of dihydrotestosterone (DHT) (0.1pM). However, in regard to tumorigenicity, E006AA prove not be tumorigenic in nude mice. A decade later (2014), the authors used a subculture of E006AA, E006AA-Par, stably transfected with *pcDNA3.1-NeoR* vector and propagated with G418-resistant cells to create a pure isogenic cell line with the specificity to be highly tumorigenic both *in vivo* and *in vitro*[251]. That cell line was named E006AA-hT. In our study, we will use the E006AA-hT, available for purchase (American Type Culture Collection (ATTC), USA) since it is the first and only cell line emanating from a primary tumor in an AA patient, most of the other cell lines (PC-3, DU-145 and LNCaP for example) being prepared from Caucasian-American patients. Like the parent cells, E006AA-hT cell lines express Androgen Receptor (AR), Glucocorticoid receptor (GR), p53, c-Met, CK-18, CK-8, and very low levels of PSA mRNA. The cell line is highly tumorigenic in both NOD-SCID and athymic nude mice and demonstrates strong malignant behavior such as invasiveness, by invading adjacent lymphovascular tissue, adipose tissue, and muscles. The cells are attached while growing with a fibroblastic aspect (Fig.12).

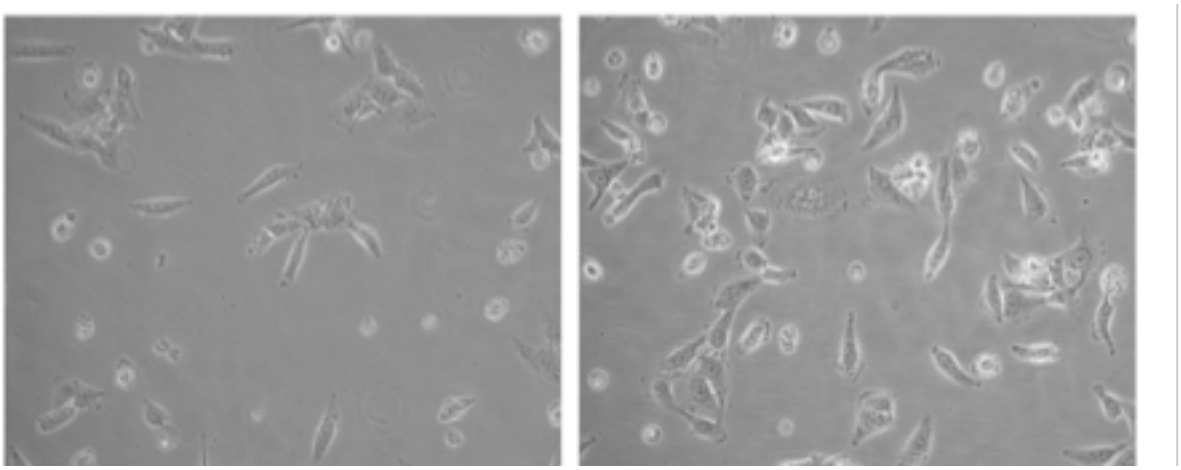

Figure 13: E006AA-hT cells. Left: low density, right High density.
Sources: ATCC website: https://www.atcc.org/Products/All/CRL-3277.aspx#characteristics

1.2-Culture of E006AA-hT

The growth medium of the E006AA-hT cell line doesn't differ from their parent and is based on Dulbecco's Modified Eagle Medium (DMEM) with a very low percentage (1 to 10%) of Fetal Bovine Serum (FBS). As suggested by Koochekpour, the complete growth medium is made of 500 mL DMEM and 56 mL Heat-Inactivated FBS [251]. The seeding density of the culture is between 1.0 x104 and 4.0 x 104 viable cells/cm^2 based on information provided by ATCC on their website (https://www.atcc.org/Products/All/CRL-3277.aspx#characteristics, Fe. the 3rd, 2017). According to the same source, the culture conditions require a temperature at 37°C with an atmosphere comprising air at 95% air and Carbon dioxide (CO2) at 5%. E006AA-hT is a fast growing cell line, easily sub-cultivable by short trypsinization (1-3 min) as 1:5 and grows to confluence within 5 to 7 days[251] . According to information provided by ATCC website, for the first 2-3 days the cells will grow slowly, then they growth reaches a more aggressive phase. It is that typical *aggressiveness* that makes E006AA-hT more suitable for our experiment

1.3- Designing Calcium Binding Peptides (CBP$_s$)

Calcium binding proteins are a family of proteins sharing in common the property to bind calcium and other metal ions [91]. They generally perform a dual function : (i) buffering calcium to protect the system against the high reactivity of the divalent cation, (ii) operating specific biological functions, through a conformational and/ or an electrical change upon binding calcium[20]. Different families of CBPs are described, their classification is generally based on the architecture of the binding site (Ex. the classic helix-loop-helix for the EF-Hand family,…) or on there distinctive chemical properties (Ex. the solubility in 100% ammonium sulfate solution at neutral pH of the S100 family,…). Marsden et al. (1990) identified three factors that are determinant to specific calcium binding (Table 1) : (i) the amino acid *composition* of the loop, (ii) the amino-acid *arrangement* of the amino-acids and (iii) the *cooperativity* between the binding sites in the event of a multiple-binding-site-protein . The architecture of several calcium binding peptides make them exceptional candidates for synthetic peptide approaches.

1.4-Peptide synthesis

Based on Marsden criteria and known calcium binding proteins, we will synthesize a number of short peptides, 10-20 amino-acids long, using proposed calcium binding sequence motif with the property to selectively and potently bind to calcium ion. The peptide will be synthesized as a C-

Terminal amide on an automated solid-phase peptide synthesizer (Intavis, Multipep model, Germany). Appropriate side chain protecting groups for various amino acids were used followed by deprotection and release from resin of the final protected peptide using a cocktail (Reagent B) containing TFA, phenol, water and triisopropylsilane as described in Reference [252].

1.4.1- Peptide purification

After synthesis, our peptide will be purified using a Reverse-Phase High-Performance Liquid Chromatography (RP-HPLC, Rainin Dynamax) protocol, based on a semi-preparative column (CSC Exsil, C18, 25 cm · 1.0 cm, Chromatography Specialty Co., St-Laurent, Quebec, Canada). Additionally, a buffer system consisting of an aqueous 0.1% (v/v) trifluoroacetic acid solution and an organic phase of ACN also containing 0.1% (v/v) trifluoroacetic acid will be used. The elution phase will be carried out through the use of a linear gradient from 15% to 60% organic phase in 105 min following a 10-min isocratic step at 100% aqueous phase (gradient A, semipreparative run) or from 0% to 60% organic phase in 60 min (gradient A, for analytical run). The flow rate will be adjusted to 2.0 or 1.0 ml/min, respectively, and the separation will be monitored by UV absorbance at 230 nm[252].

1.4.2.-Amino-Acid analysis and N-Terminal micro-sequencing

Following the purification and lyophilization, each peptide will be subjected to 24h hydrolysis in 6N HCl in a sealed tube at 110 °C in vacuo. The hydrolysed material following reconstitution in appropriate buffer solution will be analysed using the ion chromatography system (ICS-2500, Dionex, Oakville, ON, Canada) equipped with conductance detector. This will provide the quantitative analysis of the amino-acids present in each peptide. In addition each peptide will be also subjected to micro-sequencing of the N-Terminal using Procise instrument (PE Biosystem Inc., Framingham, MA, USA) and phenylthiohydantoin (PTH) chemistry. The procedure has already been described in an earlier work by Bergeron et al. [253].

1.4.3.-Mass spectral analysis

In order to confirm the identity of our peptide, we will use a matrix-assisted laser-desorption ionization-time of-flight (MALDI-TOF) mass spectrometry (Voyageur-DE Pro, PE-Biosystems Inc., Framingham, MA, USA) or surface enhanced laser desorption ionization time of flight

(SELDI-TOF) (Ciphergen, CA, USA) on gold chips (http://www.ciphergen.com) using 4-hydroxycinnamic acid or 1,2-dihydroxybenzoic acid as energy absorbing matrix [253].

1.4.4- Control peptide (CP)

A control peptide where each amino acid is mutated to its unnatural dextro variety form, with no calcium binding properties will be synthesized, purified and characterized following the same protocol as described earlier.

1.5- Operating protocol

1.5.1. Evaluating the effects of the designed peptide on the cell lines

Two (2) cell lines of each racial/ethnic group will first be grown following the technique described earlier: one for the intervention and the other one for the control. When the cells are grown healthy and attain ~80% of confluency in about 5 to 7 days of time, they will be ready for testing with the synthetic CBP and with the control peptide.

The CBP will be added to the intervention culture medium at various concentrations, varying from 1 μM to 100 μM (final concentration) in the culture medium. The same operation will be conducted in the control cultures.

1.5.2. Variables measured

To evaluate the effect of the peptides, the morphologic criteria of malignancy will be utilized as study variables. Fisher and co-workers suggested a set of cytologic criteria of which two will be of interest in the present study[254]: *Adhesion:* as measured by the degree of cohesiveness of adjacent epithelial cells . *Invasion:* defined as growth of cells without a predictable relation to pre-existing tissue landmarks established during development. To these criteria, a third variable will be added: *Growth*: We will use a cell proliferation assay to evaluate growth rate. The total cell number will be determined every 24 hrs on triplicate well. Appropriate cellular characteristics will be utilized to measure i*nvasion, proliferation and growth.*

1.5.3. Identification of the deregulated proteins profile

This is the proteomics portion of our work. Protein extracts will be obtained both from the intervention and the control cultures after a lysis of the cell using appropriate technique [255]. Polyacrylamide gel electrophoresis technique (SDS-PAGE) will be performed on these extracts and the protein profiles of the treated sample will be compared with those of the untreated and control peptide treated samples. Those proteins whose expressions are deregulated will be further analyzed and characterized using high resolution mass spectrometry. It is expected that some of the calcium binding peptide may down-regulate or up-regulate the proteins associated in the cancer with the pathways cancer pathology involving calcium.

1.6. Statistical Analysis

For measurements implying statistical data and comparisons such as the cell proliferation and invasion in *Intervention* Vs *Control* cultures, we will use SAS software version 9.3 (Cary, NC, USA). The parameters will be the mean and the standard error with a significance level at 0.05. Turkey-Kramer adjusted *t*-Test with 95% Confidence Intervals will be used for these comparisons. Data will be displayed graphically using the mean plot.

2. Results

The proteomics portion of our multi-level investigation aimed to synthesize a highly calcium specific peptide that would influence calcium metabolism in prostate cancer cell lines (Research Goal 7); evaluate and compare the responses of prostate cancer cell lines from different ethnic background : African and Caucasian (Research Goal 8) and identify the protein or group of proteins responsible for the eventual response (Research Goal 9). The Study was conducted in the Laboratory Unit of Pr. Ajoy Basak, at the Faculty of Medecine (Pathology and Laboratory Medicine Department) of the University of Ottawa. For this preliminary phase of our investigation, we were able to synthesize the desired peptide of which we provide a description here. Out of practicality and confidentiality, we will refer to the synthesized peptide as *Peptide #1*. At this stage we describe the synthesis of the peptide and provide its overall description.

2.1. Design

Several physiological proteins have been described in the literature that exhibited medium to potent binding affinity towards Ca^{+2} ion in aqueous solution[256, 257]. These included troponin superfamily proteins where it was demonstrated that a 12 amino acid peptide segment may be sufficient for binding with Ca^{+2} ion which is characterized by the presence of crucial residues such as Asp at position 1 (+X of the coordinating position of the calcium), Asp or Asn at position 3 (+Y), Gly at position 6, Ile at position 8, and Glu at position 12 (-Z) [256]. In a later study the same authors designed a peptide from skeletal troponin C within the sequence from residue (103-115) [258]. This is present within the site III of rabbit skeletal troponin C [258]. Other proteins which exhibited strong Ca^{+2} binding activity in physiological systems included Calcyclin, Alpha-cardian actin, Calbindin, Calmodulin and Porcine plasma protein[256, 257] . Based on the specific segment of these proteins, known to be associated with Ca^{+2} binding property, we designed several short peptides that we expect to show Ca^{+2} binding affinity. The amino acid sequences and sizes of these peptides are depicted in Table-VII. We also decided to attach a fluorescent tag (in this case 5-Carboxy fluorescein) at the amino terminus of each peptide for easy visualisation and fluorescence intensity study following binding with Ca^{+2} ion.

<u>Table VII</u>: List of peptides with potential Ca^{+2} binding property designed from various physiological proteins with known Ca^{+2} binding properties and based on Marsden's review. ***Note: each amino acid is indicated by its single letter code***

Peptide No Amino acid sequence	Calculated Mol. Wt.	Precursor protein
1. 5-Carboxy-Fluorescin-GTNYDEG	1095 Da	Calcyclin
2. 5-Carboxy-Fluorescin-GYSFVTTAER	1470 Da	Alpha cardiac actin
3. 5-Carboxy-Fluorescin-ITGQSLGYGFVNYIDPK	2228 Da	
4. 5-Carboxy-Fluorescin-$(XYZ)_n$ (With n = 1-40 and X=D, E, N, A, Q and Y/Z=A, S, T, S)	?	Designed based on previous publications
5. 5-Carboxy-Fluorescin-VSGVEDVN	1175 Da	Porcine plasma protein
6. 5-Carboxy-Fluorescin-DKDGDGFIDFEE	1743 Da	Designed
7. 5-Carboxy-Fluorescein-DADGNGTIDFPE	1590 Da	Calmodulin

Note: Da = Dalton, Mol. Wt. = Molecular weight

2. 2. Chemical Synthesis, Purification and Characterization of the peptides

All peptides were or will be prepared by solid phase Fmoc-based chemistry using our Intavis automated solid phase peptide synthesiser and a 96 well plate. The detail procedure, type of resin, time for each coupling, coupling reagent (HATU), nature of amino acid side chain protecting group as well as the chemistry steps were described in detail in reference [257, 259]. In the last step while the protected peptide is still anchored on the resin, it is attached to 5-carboxy fluorescein via its free amino terminus. In the end the peptide is released from the resin and fully de-protected using reagent K as described in reference[260, 261]. Briefly the materials and chemistry used are described.

2.3. Materials

All Fmoc amino terminal protected amino acids (L-configuration) with additional side chain protection as needed, peptide coupling reagents namely HBTU, PAL-PEG-PS resin for peptide synthesis, organic solvents such as Acetontrile (ACN, HPLC grade) and Dimethyl formamide (DMF, analytical grade) were obtained from Bachem Inc (Torrance, Ca, USA), Calbiochem Novabiochem Inc, (San Diego, Ca, USA), Neosystems Inc, (Strasbourg, France) and PE-Biosystems (Foster City, Ca, USA). TFA and all reagents such as phenol, TIS and EDT constituting Reagent B [260, 261] for peptide deprotection and its cleavage from resin were purchased from Aldrich-Sigma Chemical (Milwaukee, USA). TCEP [Tris (2-carboxy ethyl) phosphine], Iodoacetamide as well as all other coupling agents and organic solvents were bought from Sigma-Aldrich, VWR or Fisher companies. Surface Enhanced Laser Desorption Ionization (SELDI) time of flight (tof) mass spectra (MS) were recorded using Voyageur (PE-Biosystems, Framingham, Ma, USA) and Ciphergen Protein Chips (Fremont, Ca, USA) respectively. The corresponding mass spectra plates, re-usable gold plates for SELDI and stainless plates for MALDI were purchased from the respective companies. 1-Cyano 4-hydroxy cinnamic acid (CHCA), 2, 5 Di-hydroxy benzoic acid (DHB) and Sinapic (Aldrich-Sigma Chemical) were used as energy absorbing matrices for low and high molecular weight compounds respectively.

2.4. Methods and Procedure

Peptides were synthesized on an automated solid-phase peptide synthesizer instrument (Intavis, Multipep model, Germany) using Fmoc (Fluorenyl methoxy carbonyl) mediated chemistry and HBTU [N,N,N′,N′-Tetramethyl-O-(1H-benzotriazol-1-yl) uronium hexafluorophosphate], HOBT (1-Hydroxy benzotriazole) in presence of DIEA (N, N'-diisopropyl ethyl amine) as coupling reagent [57]. The following amino acid side chain protecting groups were used: Pbf (2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl) for Arg; tBu (tertiary butyl) for Ser, Thr, Tyr, Asp and Glu residues; Trityl (triphenyl methyl) for Cys, His, Gln and Asn and Boc (t-butyloxy carbonyl). The synthesis started from carboxy (C-) to amino terminus (N-) end on an unloaded Fmoc-protected tentagel [a PAL-PEG {poly amino linker poly ethylene glycol} cross linked to PS (polystyrene)] resin. Following the end of synthesis, peptides were cleaved off from the resin and fully deprotected at the same time by 3h treatment with Reagent B consisting of 90% TFA (Trifluoroacetic acid), 2.5% phenol, 5% water and 2.5% TIS (Tri-isopropyl silane) [g, h] for 3h at ambient temperature. The crude peptides thus obtained were purified by RP-HPLC and fully characterized by SELDI (Surface Enhanced Laser Desorption Ionization) -TOF (Time of flight) Mass Spectrometer (MS) (Protein chips, Ciphergen, Fremont, CA, USA) using CHCA (α-cyano 4-hydroxy cinnamic acid, DHB (2, 5 dihydroxy benzoic acid) (both from Sigma-Aldrich Chemical Co, Milwaukee, WI, USA) or SPA (Sinapic acid) as an energy absorbing matrix.

RP-HPLC: The crude peptide obtained from synthesiser was purified by Reverse Phase High Performance Layer Chromatography (RP-HPLC) using C_{18} Silica gel analytical column (Varian, 10 x 25 cm size). During RP-HPLC purification, proteins were separated using a linear gradient of Solvent B from 10% to 90% in Solvent A [Solvent B =0.1% TFA in ACN and Solvent-A = 0.1% TFA in water]. Fractions were collected and analyzed as the elution was monitored on-line by UV detector with wavelength fixed at 230 nm. Peaks were collected, lyophilized and subjected to mass spectrometry for their identifications[260, 261].

So far only one peptide namely **peptide#1** was successfully synthesized and purified as well as fully characterized. This peptide was purified by RP-HPLC. The peak eluting at Rt = 50.04 min was collected and analyzed by analytical. RPHPLC chromatogram also confirmed its homogeneity. Seldi tof mass spectrum of the purified peptide showed a single peak at m/z 1096 $(M+H)^+$ consistent with its calculated molecular weight (See Table VII). The pure peptide was

finally characterized by amino acid analysis, and by digestion experiment with endoproteases Lys-C enzyme which cleaves peptide bonds specifically after Lys residue.

2.5. Evaluating the Calcium binding properties of Peptide#1

To avoid competition with EDTA we used instead 1% dilution of purified peptide #1 which was progressively added to 1.5% calcium chloride ($CaCl_2$) solution. The calcium binding properties of **Peptide #1** was evaluated by the formation the calcium adduct as following treatment with progressively diluted sample of the peptide. The extent of adduct formation was analysed in a semi-quantitative manner using mass spectroscopy technique associated with a standard peptide sample of high purity.

Chapter 7: Preliminary Synthesis and Discussion

In this portion we combine the preliminary findings of Phase I and II and suggest socio-ecological models of prostate cancer disparity. This synthesis was presented at the American Association for Cancer Research (AACR) 10th Annual Symposium on Cancer Disparities[3] in September 2017.

1. The Calcium Principles of Tumorigenesis

Based on the findings from Chapter 3, it appears that two essential notions that very well summarize calcium homeostasis are *(i)* its differential concentration in the various compartments and sub-compartments of the cell, and (*ii)* the tireless and innate effort of the cellular machinery to restore these baseline concentrations whenever they are perturbed. Additionally, unlike the other minerals, calcium metabolism is closely related to external environmental factors, this in two ways: firstly, as mentioned before, the quasi totality of internal calcium derives from nutritional sources, hence the importance of diet. Secondly, calcium metabolism, as we have shown is *cooperative* with calcitriol, the active form of Vitamin D, also referred to as 1,25 dihydrocholecalciferol or $1,25(OH)_2\ D_3$. Between 80 to 90 % of body Vitamin D in humans, is produced in the skin through dermal photosynthesis under the effect of solar ultra-violet B exposure. Thus Calcium metabolism borrows greatly from strong external factors: diet and exposure to sunlight in particular. We have established the calcium perspective of tumorigenesis views cancer as the *over survival* of a cell from a chronically injured tissue, through evasion from all life-controlling mechanisms. The core mechanism underpinning that *over* survival is a self-sustained *defective* calcium signal in the different cellular compartments and sub-compartments. The *primum movens* of the cascade is a sub-lethal increase in intracellular calcium, and the subsequent implementation of correcting mechanisms. In that approach tumor initiation follows two phases: a phase of calcium increase or phase I, later followed by a phase of cell habituation to low calcium levels or phase II (Fig.7). One of the key advances in our approach is the integration of the existing major cancer pathways to the theory which classifies them in two groups: the calcium store dependent pathways and the calcium wave dependent pathways with a

[3] KADIO, B., Yaya, S. H., Basak, A., Gomes, J., Djè, K., & Mesenge, C. (2018). Abstract A35: An integrative model of cancer disparities based on the calcium molecular theory of carcinogenesis.

final common effect on proto-oncogenes and tumor suppressing genes expression. As a consequence, three general principles can be enunciated to summarize the role of calcium in tumor development:

Principle 1 : Deriving from calcium versatility, each body tissue has a preset physiological range of calcium concentrations in its different cellular compartments and sub-compartments for optimal functioning of the cells of that tissue.

Principle 2: Any environmental event, or conjunction of environmental events, the biological translation of which tends to accrue the influx of calcium into the cells of a given tissue, beyond their normal physiological levels, on a chronic and repetitive basis, has the potential to initiate cancer in that tissue. The same rule will apply with cellular calcium flux reducing stimuli. This can be referred to as environmental hyper or down regulation of calcium.

Principle 3: In order to insure swift adaptative responses to environmental signals, cells from the neuroendocrine tissues have physiologically narrower ranges of calcium concentration in their different compartments and sub-compartments concentrations.

It is the ensemble of those molecular principles that help connect prostate cancer risk factors and the expression of the disease in one specific population. But before discussing that point, it will be useful to comment on a new notion recently developed in the cancer literature : the very new concept of transgenerational epigenetic inheritance (TEI)[262].

2. Traditional Nutritional Calcium Intake Patterns in Subsaharan African males

From the Pilot Study of the APCS, it came that the traditional African diet as far as is calcium concerned diverges greatly from Western diets both qualitatively and quantitatively. There is also a divergence in terms of cooking practices. The Subsaharan intake of calcium derives from seasonal tropical plant foods either sun-dried or fresh. A generous amount of fruits and vegetables such as papaya, oranges, passion fruit, banana or okra to name a few are part of the daily diet as evidenced in the study. Another important source on calcium is whole sun-dried or smoked fish. While smoking as a cooking practice has its own issues, in terms of increased aromatic components in diet[68], and exposure of women to heat, sun-dried whole fish provide a substantial amount of

calcium. The intake patterns of calcium in a Western type of diet mostly derive from dairy products, fruits and vegetables are just accessory. Stated otherwise, when Subsaharan Africans are placed in a Western type of environment, the first generation goes unto a radical change in calcium intake patterns with the potential to affect the cellular exchange mechanisms described earlier. As for quantity it is traditionally believed that African populations have a low calcium intake. Like we said earlier such statement should be nuanced as they are based on dairy consumption levels. As we have shown, some dried plants have a higher content in calcium than dairy, and the so called osteoporosis paradox, whereby Subsaharan women has a low osteoporosis rate yet with a low calcium intake might just be apparent. Our key point here is that the traditional subsaharan diet especially for a the vital element of calcium, is vey different in many regards with the Western type. We will see in the next paragraphs how this change combined with other risk factors in the broader context of health determinants, turn into a biological stress that contribute to prostate cancer.

3. Health Determinants and Prostate Cancer Risk Factors

There is a difference between *determinants* and *risk factors*. Health determinants refer to underlying characteristics of society that ultimately shape the health of individuals and communities, whereas risk factors explain variation in risk around the overall population mean level[72]. In other words, determinants shape the health status of a population, selecting those the most susceptible to be sick, and risk factors will determine not only disease type and patterns, but the individuals within the selected population who will actually be affected. We apply this distinction to prostate cancer, and summarizes in Table VIII, established health determinants using the framework suggested by Public Health Agency of Canada (PHAC http://www.phac-aspc.gc.ca/ph-sp/determinants/determinants-eng.php) and the deriving currently known risk factors of the disease. We have also suggested some references for further consultation. It's important to distinguish between risk factors that influence incidence and those affecting mainly outcomes. Study suggest that the racial differential of prostate cancer disappears after radical prostatectomy, relapse rates are the same[263]. It's also very interesting to note while the risk factor of « *family history of prostate cancer »* can be connected to *history* as an established health determinant, *race/ethnicity* and *age* the two other well established risk factors can only be connected to *genetic* as a health determinant. But we have already mentioned in the first chapter that over 80% of the cases were not related to any known single nucleotide polymorphism.

Therefore race/ethnicity and age could be seen as proxies of other determinants, which we will discuss in the coming paragraph after we discuss the role of molecular calcium.

Table VIII : The determinants of health and Prostate Cancer risk factors

Socio-ecological Level	Health Determinants	Prostate Risk Factors	Identified Mechanism
Individual	History	Family History of Prostate Cancer	Transgenerational Inheritance
	Socioeconomic Status (SES)	Occupations such as Farming, Industrial	Exposure to Chemicals, Pesticides
	Anthropometrics	BMI/ Adult Height	Growth Factors - Androgenic Hypersecretion
Social	Health Service	Knowledge and Access	Delays Diagnostic - Related to Fatal Outcomes
	Culture	Diet:Dairy products, Processed and Red Meat, Cooking Practices	Growth Factors - Androgenic Hypersecretion - Chemicals
	Behavior	Sexual behavior (STI), alcoholism, Physical activity	Chronic inflammation-Influences outcomes
Environmental	Place	Exposure to sunlight	Deficit in Vitamin D
	Industrialization	Exposure to polluant	Androgenic hypersecretion

4. Transgenerational Epigenetic Inheritance

As defined in Chapter 2 and briefly described in the last paragraphs of Chapter 4, the epigenetic paradigm refers to the expression of phenotypic traits non supported by either a somatic or germline mutation in the cellular DNA sequence. The cellular changes are instead imprinted in the cells by the environment to which they are exposed through different cellular mechanisms [264]. *Environment* here is to be understood in the broader sense to include physical, social and behavioral interactions. In relation to cancer, epigenesis refers to the *silencing* of tumor-suppressing genes and/ or *overexpression* of proto-oncogenes under external pressure [265]. Evidence is just now surfacing that in humans and in other species as well, behavioral and environmental manipulations affecting parents can induce changes potentially transmittable to the offsprings for the following several generations, in a non-mendelian inheritance manner. This process has been termed *transgenerational epigenetic inheritance* (TEI) and was first described in 1956 by Brink and coworkers [266]. Several environmental stimuli of the parental lines susceptible of triggering TEI have been described. They are various in nature and all based on key parameter : *prolonged dramatic change*. Triggers of TEI hence include changes in the physical environment such as in temperature [267], in exposure to sunlight[268], in oxygen availability[269]. Cultural and social factors have been described as well including changes in parental care[270], in diet [271], in exercise[272] but also traumatic experiences [273]. Molecular mechanisms involved in these non-mendelian types of inheritance are currently being explored but it is already established that well-known epigenetic mechanisms are at play. Indeed, different forms of DNA methylation processes are said to evade the *demethylation* process at conception and have been correlated to TEI by several studies at both molecular and epidemiological levels. For example the carbon in position 5 of Cytosine (5mC) has been correlated to differenciation, development, aging and diseases[262]. Classic examples are cited in the literature and derive from correlative epidemiological evidence suggesting the connection between environmental signals encoded in DNA methylation processes and passed down to the offsprings. For example Heijman and collaborators assert that prenatal exposure to famine during the Dutch Hunger Winter (1944-45) was associated with lower cytosine methylation on the imprinted insulin-like growth factor 2 (IGF2) gene. Similarly, a decreased cytosine methylation has been described in FK Binding Protein 5 (BP5) a gene related to stress levels, in the offsprings of Holocaust survivors[273]. Other molecular mechanisms or agents include non-coding RNAs, Histone modifications, chromatin remodeling, proteins and

microbiota[262]. The extent to which TEI can be suspected in prostate cancer disparities comes from the *familial history of cancer* as a risk factor. It is established that males born from parents with either breast or prostate cancer has an excessively increased risk to develop prostate cancer [61]. It also comes from the persisting racial gap in spite of voluntary public policies aimed at increasing access to healthcare for the most vulnerable[274]. Although more specific mechanisms are yet to be elucidated, a series of work recently published provide mounting evidence that TEI mechanisms might be at play in prostate cancer disparity. In 2015 Yanyuan and coworkers conducted a strong state-of-the-art review of epigenetic mechanisms involved in both Breast and Prostate malignancies [275]. The authors identified some key mechanisms that we briefly describe here. The first mechanism relates to DNA methylation. Studies have established that 85% of prostate cancer cells and cell lines exhibited hypermethylation of CpG islands of some genes[276]. CpG sites are high frequency DNA regions where cytosine nucleotide (C) is followed by a guanine nucleotide (G). The list of genes methylated in a specific case is referred to as the *methylation profile* or *patterns*. Available research suggest that the methylation profile of a given prostate cancer case have predictive values in terms of tumor aggressiveness [277] and the positivity of the tumor expression of androgens receptors with tremendous impacts in terms of diagnostic and prognosis. Another epigenetic mechanism is the histone modifications affecting androgen activity. Empirical experiments suggest that Histone protein acetylation and chromatin remodeling affect both qualitative and quantitative expression of androgen receptors (AR) and activity in the prostate cell. Inhibition of Histone deacetylase enzyme (HDAC) activity significantly increased endogenous AR activity, as well as the prostate-specific antigen (PSA) in LNCaP cells. Are those epigenetic mechanisms racially differentiated ? Most epidemiological prostate cancer studies have a common weakness in their design : sampling. While AA men have a two-fold incidence compared to men from other race/ethnicities, to very few exceptions they are in smaller number, at the best equal, in most studies. The issue has been raised by several authors recently[278]. Nonetheless, available studies suggest that there is a higher methylation rate in African Americans compared to Asians and to Caucasians[279]. In 2015, Bi-Dar Wang and colleagues used an integrative genomics method based on non-coding micro-RNA (miRNA) and Messenger RNA (mRNA) profiling, miRNA target prediction among other techniques to map the interactions between miRNA-mRNA associated to prostate cancer disparities. The authors identified AA-specific enriched miRNA-mRNA parings that activated oncogenic pathways in AA prostate cancer cases. They also identified pairs that were uniquely associated to tumor aggressiveness in AA males[280]. It then appears that the epigenetic mechanisms involved in prostate tumor development might be racially differenciated along the lines

of different male populations for both DNA methylation, chromatin remodeling and miRNA pairing. The question then becomes are these mechanisms inherited or transmitted from parents to offsprings without following the Mendelian model? What we can refer to as *The Vinclozolin Experiment* conducted by Klukovich and coworkers and reported in July last year (2019) is quite informative on the question. Vinclozolin ($C_{12}H_9Cl_2NO_3$) sold as *Ronilan* , *Curalan*, *Vorlan* or *Touche* is a common fungicide used for disease control in agriculture. Documents attest that it has been circulating since the 1980s. It has a long and not always clear history of regulation in the US, and exposure to the product and to its metabolites comes through dietary (food and water), non-dietary and occupational pathways. It's established to interfere in a competitive manner with main hormones receptors including androgens, progesterone and estrogen[281]. Klukokovich et al. exposed gestating female rats (F0 generation) to vinclozolin during E8-E14 of gestation. Then F1 generation offspring were bred to produce the F2 generation, which in turns were bred to produce the transgenerational F3 generation. The Authors report that F3 generation vinclozolin lineage males at 12 months of age had an increased incidence of prostate histopathology and abnormalities compared to the control lineage. They also isolated prostate epithelial and stromal cells from F3 generation 20-day old rats, before any sign of prostatic disease for DNA and RNA analysis. Results evidenced transgenerational changes in gene expression, noncoding RNA expression, and DNA methylation in both cell types. The author concluded that *ancestral exposure to vinclozolin at a critical period of gestation induces the epigenetic transgenerational inheritance of prostate stromal and epithelial cell changes in both the epigenome and transcriptome ultimately leading to prostate disease susceptibility.* If the focus in that study was on environmental toxicants, it is likely that other stressors emanating from the social, ecological and individual environment in parents might as well lead to epigenetic changes that are passed on to the children and grand-children. We already mentioned in Chapter 4 that calcium underpins the molecular pathways of epigenetic mechanisms as supported by available epigenetic therapeutics [169]. It's therefore now possible to suggest an integrative model combining prostate cancer risk factors, calcium molecular principles and transgenerational epigenetic.

5. A Socio-ecological Model of Prostate Cancer Disparity

5.1. Key Concepts and Constructs

Why African ancestry, family history and age are the most firmly established risk factors of prostate cancer? The short answer would be *change.* A more detailed answer needs to go through a more in-depth historical analysis and combine biological and environmental factors . The first generations of African Americans along the Routes of Slave Trade and of the subsequent African Diaspora in the Western Hemisphere, say *generation F0*, have been exposed to protracted and radical changes that have transformed their new environment into a permanent stressor. New diet and nutritional patterns affecting key body function element such as calcium, abrupt rupture of social relations and interactions, new patterns of sunlight exposure, temperature changes, « *occupational* » exposure to environmental toxicants, permanent stress for survival. Let's refer these changes affecting F0 as *dramatic environmental stressors* (Fig.14). The cumulative effect of these stressors, the permanent and chronic threat they represent would result in new biological mechanisms of *survival* supported by calcium metabolism, because of the three principles of calcium cellular metabolism mentioned earlier : increase in cellular calcium influx on a chronic basis with a resulting increase in cellular compartments and sub-compartments concentrations mostly in the Neuroendocrine Systems (NES). The NES controls life preservation mechanisms and survival. The aim here is therefore to *survive* to the new stresses and to better adapt to this *new environment.* This results in a constant *suppression-survival* interaction: the higher the suppression, the higher the survival. This calcium-based exchanges ultimately induce epigenetic modifications in the NES and accessory in other systems as well. Cortisol, Androgens, Progesterone, Estrogens,…levels will all be influenced along with other hormones levels. When the offsprings of F0 develop, say *Generation F1*, transgenerational epigenetic mechanisms are activated, and high defense systems are passed down to F1. Furthermore the persistence of the *suppression-survival conflict* creates a self-sustained cycle only deepening the survival mechanisms, delaying the correction processes and imprinting them deeply in the nucleotides but not in a mutational manner, in order to create a cell memory. But the sustained cellular modifications affecting both the NES and certainly many other calcium dependent biological systems, result over the years in the silencing and/or over expression of a series of genes. At the phenotypic level this will translate in the cellular proliferation of the most *exposed or vulnerable* neuroendocrine organs of which the prostate gland is among the first. When the first

SEER studies observing the high incidence of prostate cancer in men from African ancestry, not only they are describing the inherited phenotypic traits of say, *Generation Fn*, but it might even be that the disease is only *the pick of the iceberg*. High blood pressure, trauma, post traumatic stress, Anxiety, psychosis,…potentially all diseases directly related to the NES might be part of the picture. In this perspective, and as counterintuitive as it could be, *Race/Ethnicity* and *family history of (prostate) cancer* are proxies of one and the same determinant of health: History and not genetic. *Age* is not a risk factor *per se* but rather should it be seen as a risk *indicator* or risk *marker*, simply setting the time needed for biological mechanisms to reach their *maturation* and trigger prostatic disease. As for the worse outcomes of the disease reported in African males, they too borrow from those two same molecular and environmental mechanisms but at the only difference that contrary to the *incidence model* in which one mechanism leads to the other, in the *outcome model* the two work separately. Not only calcium ion orchestrates tumor initiation under the pressure of upstream factors, the divalent cation is also active in tumor promotion and progression, under current environmental stressors such as diet and other factors. Thus, a historically and transgenerationally ill calcium signal has the potential to also fasten malignancy. On the other hand, the persistence of social structures, norms and culture affecting *invisible minorities* in countries where social inequalities are rampant, ultimately impacts, the trust, the care seeking behaviors and access to appropriate health services for individuals, delaying time of diagnostic and treatment which could be ultimately detrimental and increase mortality. In the next paragraphs we suggest two schemas outlining the two models : the Increased Incidence Model (IIM) and the Worse Outcomes Model (WOM) (Fig.14 and 15), followed by a discussion on the coherency of our approach with current population health theories.

5.2. Schematization

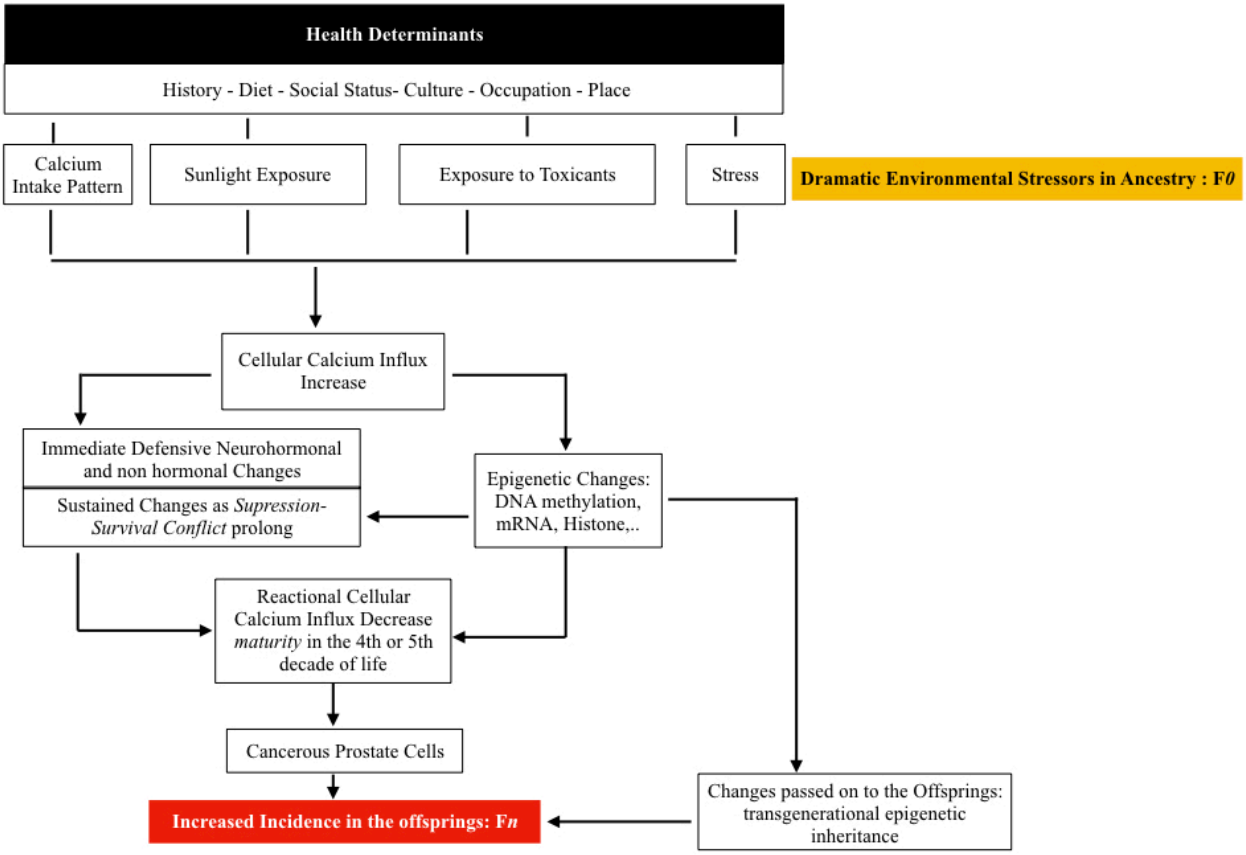

Figure 14: Socio-ecological mechanist model of prostate cancer incidence disparity (Increased Incidence Model)

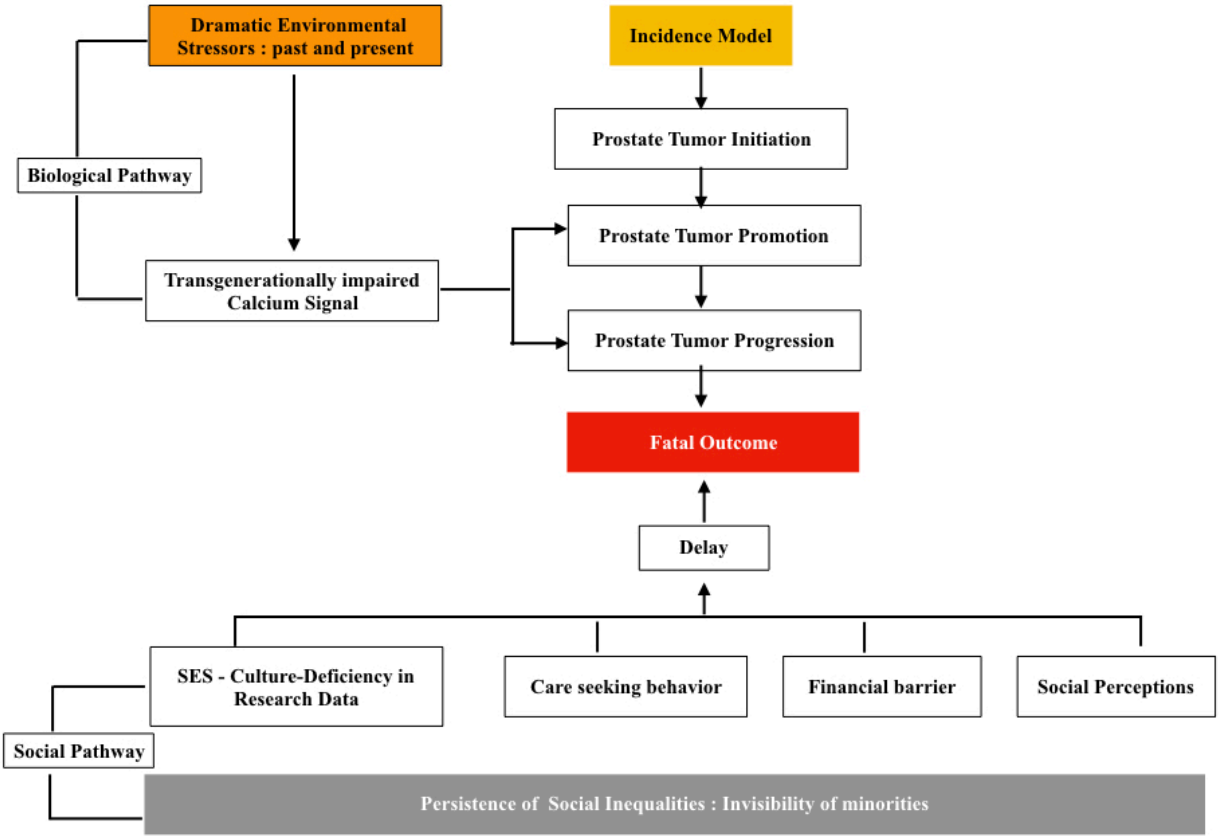

Figure 15: Model of prostate cancer outcomes disparity (Worse Outcomes Model)

5.3. Coherency of the Models with Population Health Main Theories

One main inquiry of Population Health as a research field is the positive identification of the pathways whereby social inequalities turn into disease. That is how the differences in social social status « *get under the skin ».* Over the years four main theories have been suggested by different authors from broad array of disciplines. The first hypothesis is known as *the psychosocial pathway,* and it was developed by Marmot and Wilkinson : in that approach neuroendocrine factors mediate the stress imposed by the social conditions and ultimately leading to a differentiate expression of disease with a pic of incidence in those undergoing the social stress [282]. It's such an approach that is commonly suggested to explain the findings of the *Whitehall Studies* and to explain unhealthy behaviors such as smoking. Another pathway is known as *Lynch's neo-material hypothesis* where health disparities is rather a resultant of a differentiated uptake and use of social infrastructures, public or private having a positive impact on health status such as healthcare facilities, physical activities areas, schools, housing, neighborhood… [283]. Lynch gives the metaphor of passengers in air travel: those traveling in first class have a far more enjoyable trip and arrive at destination more rested and in better health than those traveling in economy. The third view relates to *social capital* . It was suggested and developed by Kawashi, among others, in that theory, differenciated health status is a product of an impaired social network in which a diminished social cohesion induces poor individual to lack the necessary social support needed to avoid the worse forms of disease, or to live in such an isolation that they become vulnerable to any type of disease [284]. Finally the last theory suggested to explain how disease get under the skin is the *embodiment* also known as the *socioanthropological pathway*. The concepts was first enunciated by Nancy Krieger in a research published in 1999. In an attempt to define key concepts, methods and and measures that should govern the investigation of the effects of racial discrimination upon health. Krieger anticipated that both economic consequences of discrimination and accumulated insults arising from everyday and at times violent experiences of being treated as a second-class citizen, at each and every economic level would ultimately be embodied and turn into ill-health via direct and indirect mechanisms [285]. But Nguyen and Peschard take a step further to explain from a socio-anthropological view how social inequalities are embodied across societies and times. For theses authors, the body is not a mere and passive recipient of social inscriptions. Instead, the body is the place of a dynamic resistance to social violence such as colonialism, slavery , patriarchy and capital among others. The forms of that resistance are varied and time dependent according to the authors. For example, in premodern societies *embodiment* was more in the form of rituals, scarifications and other form of

body expressions, but in modern and postmodern societies embodiment is deployed through more subtile biological mechanisms translating in terms of differenciated morbidity and mortality rates between groups at the different position of the social ladder [286]. We can note that both models as suggested in this research (The Increased PCa Incidence Model and the Worse PCa Outcome Model) combine to various degrees those different pathways suggested by the Population Health scientists. In our Increased Incidence Model, we assert that the *Dramatic Environmental Stressors* impact the neuroendocrine system (NES) through impairing cellular calcium influx. The idea of an internal impact on the NES from environmental factors through calcium metabolism is also supported by Carafolli and Krebbs in their work on calcium homeostasis published in 2012 [287]. The authors elaborated on the interaction using a schema that comprised four main actors : Calcium uptake, Internal Calcium, Hormones and glandular tissue working in a closed loop forming a feedback or retro-control system (Fig.16). Upon an external stimulus, say increased calcium uptake, an ultimate consequence will impact the internal calcium component of the loop which in turns will impact the glandular tissue and trigger hormonal secretion tending to decrease that internal calcium. Our model is that if such mechanism is prolong for a long period of time, glandular cells will develop adaptative mechanisms leading to uncontrolled proliferation in the glandular tissue. Such an approach is coherent with the Marmot-Wilkinson *biopsychosocial pathway* mediated by neuroendocrine factors. Thus, calcium metabolism is the connector between external factors and internal responses supported mainly by the NES. Our Increased Incidence Model also echoes Nguyen and Peschard *socio-anthropological pathway* in which, as we mentioned earlier, differenciated expression of disease is the embodiment of a resistance to the violence of social inequality. That is to say that *t*he body actively organizes its own resistance mechanisms to societal violence in order to survive. The key concept of our Increased Incidence Model is *survival*. The *Dramatic Environmental Stressor* tends to suppress life through a series of environmental events that would biologically translate into a chronic increase in cellular calcium influx. Some cells will die, but a surviving cell react to that increase through a series of cellular adaptation that would result in the decrease of calcium influx ultimately beyond a certain threshold, leading to tumor initiation. The *survival approach* is also confirmed by Hanahan and Weineberg in their description of cancer cells hallmarks. One of the key features of cancer cells is their *innate ability to survive* as manifested in their resistance to any form of apoptotic mechanism [76]. Also, our *Worse Outcomes Model* aligns with Lynch's *neo-material hypothesis* and Kawashi's *social capital theory* as well. Indeed, the Outcome Model is based on two distinct but cooperative segments : (*i*) a biological segment in which transgenerationally ill calcium signal will precipitates the fate of the affected

prostate gland, (*ii*) the model also comprises a social segment in which the prolongation of historic and cultural perceptions about disease and healthcare, combined with the reminiscence of structural obstacles from the legacy of past discrimination interplay to reduce the social capital of men from African ancestry in Western countries and also limits their uptake of the neo-material conditions favorable to health. The perceptions and economic limitations also delay their time in sicking care at the onset the prostatic disease. The *a posteriori* illustration of this effect of social and neo-material influences is that prostate cancer outcomes become similar, between European African and African American males after treatment or when adjustment is made in epidemiological studies to some variables such as access to care, socio-economic status. Thus both the Increased Incidence Model and the Worse Outcome Model of PCa in men from African descent as suggested in this study are consistent with our current knowledge of pathways whereby social inequalities *get under the skin.* In the the next paragraph we provide an illustration of our model using Endocrine Disruptors as an illustration of the Dramatic Environmental Stressor, after which we discuss the limits of our approach and what are the next steps for better explore the new theory.

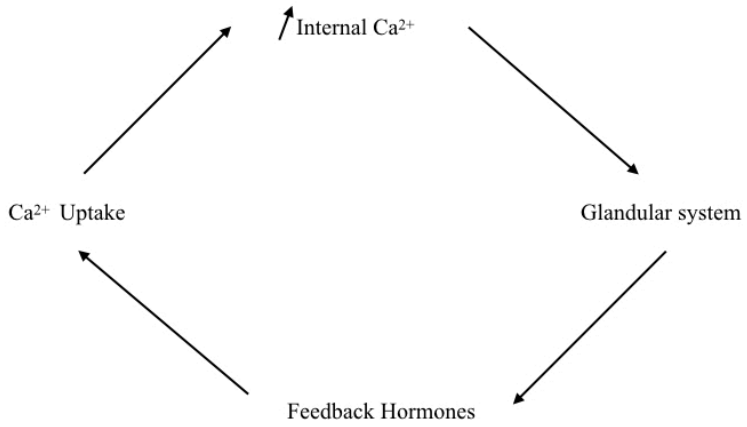

Figure 16 : *Carafoli-Krebbs Model of the environment-hormone calcium centered relationship.*

5.4. Illustration : Prostate Cancer in Jamaica and the West Indies and in Virginia.

5.4.1. Prostate Cancer in Jamaica and the West Indies

We provide here two examples of the calcium-centered theory of prostate cancer disparity. In 1998, a study in Jamaica reported the highest age-incidence specific in the world[288]. It was estimated at 308/100 000 and involved mostly the men from an African decent. Studies have been conducted to help explain what factors could account for such a high incidence. It has been suggested that Vitamin D deficit might be at play[289], however a case control study by Jackson and co-workers in 2008, failed to evidence any association with calcium and Vitamin D intakes levels. Interestingly though, the study found a positive association between mean serum calcium concentrations and prostate cancer. Serum calcium was higher among cases (2.32 ± 0.19 mmol/L) than controls, (2.27 ± 0.30 mmol/L) (P = 0.023). Similarly, Serum 25(OH)D concentration also showed a positive association with PCa (OR, 1.23; CI, 1.01–1.49 per 10 ng/mL). The authors were inconclusive about the possible mechanisms underpinning their findings. How does our model apply to Jamaica ? The best situation that explains the life of Jamaican men from African descent is poetically described by American poet Howard Pyle : « *Jamaica, like many another of the West Indian Islands, is like a woman with a history. She has had her experiences and has lived her life rapidly. She has enjoyed a fever of prosperity founded upon those incalculable treasures poured into her lap by the old time buccaneer pirates. She has suffered earthquake, famine, pestilence, fire and death: and she has been the home of cruel merciless slavery, hardly second to that practiced by the Spaniards themselves. Other countries have taken centuries to grow from their primitive life through the flower and fruit of prosperity into the seed time of picturesque decrepitude. Jamaica has lived through it all in a few years.* » *in* Jamaica New and Old" in Harper's New Monthly Magazine, January 1890, page 23.

The author would have certainly tempered its enthusiastic prose would he has known that the stress of this tormented history disproportionately bore by the African population of the Island, would later translate into one of the world highest incidence of prostate cancer in their offsprings. But to this historical background, another important factor should be added. The Proceeding of the 26th West Indies Agricultural Economics Conference (Caribbean Agro-Economics Society) published in May 2007 and entitled « *Pesticide Use and Human Health in Northwestern Jamaica »,* the abstract

so reads *: « A number of studies have detected high levels of pesticide residues in surface water and aquatic life in Jamaica and acute pesticide poisoning is believed to be widespread there. Despite efforts by the Jamaican government to create awareness of the dangers of pesticides and adopt safe a pesticide disposal method, many farmers still display poor pesticide handling and disposal practices. The objectives of this study were to 1) describe pesticide use by farmers in northwestern Jamaica including inappropriate methods in pesticides handling and disposal, and 2) determine whether farmers' perception of the mode of bodily entry of pesticides affects their method of disposal. Farmers in Westmoreland, St. James and Hanover were surveyed using an investigator-administered instrument. Although 96% of farmers had some form of formal education, 75% had received no training in the use of pesticides. Only about 15% thought that crop yields and quality could be maintained without the use of pesticides. Only 29% believed that pesticide use posed a health risk, while 91% thought that pesticide use was an environmental hazard. Less than 45% of farmers used safety gear (gloves, masks, goggles) in handling pesticides although 65% always used special clothing. A fair proportion of farmers burn, bury or dump unused pesticide or empty pesticide containers in the bushes. Farmers' disposal methods were influenced by their perception of the ways that pesticides enter the human body. Thus, a large percentage of farmers in these parishes use pesticides inappropriately and are exposed to pesticides in handling. Disposal of unused pesticides and empty pesticide containers pollute the environment and most likely expose others. Measures should be taken to educate farmers, to provide protective gear at an affordable price, and to implement a clear and consistent method for collection of unused pesticide and empty pesticide containers.* » [290] page137.

Interestingly, converging data suggest that the Caribbean island of Jamaica records one of the highest contamination in environmental toxicants per square meter in the world. Detection of insecticide residues have been found in 14 of 17 rivers, 4 of 7 natural springs, and 8 of 13 wells in in the country [290]. Hence, it appears that the combination of wide use of Environmental Endocrine Disruptors in the form of pesticides and insecticides, in population historically suppressed has culminated in the highest incidence in prostate cancer in the world in Jamaican males from an African descent. The *dramatic environmental stressors* involved here are social status, stress and environmental toxicants. It appears that it is such a similar mechanism that is still at work in the whole Caribbean Islands, where territories like French Guyana, record very high incidence rates along with what is now known as the *Chloredecone scandal* [291].

5.4.2. Prostate cancer in Virginia

In 2014, Virginia's prostate cancer statistical data displayed one of the worst disparities in the US with a 1.97 incidence and 2.4 mortality ratios (national respectively 1.8 and 2). Incidence distribution in the 35 health districts presented a clear east-west gradient. Crater (179.0 cases per 100,000 men), Hampton (174.3), and Chesterfield (163.2) all located in the Eastern and Central portions of the State recorded the highest incidences whereas Lenowisco (60.8), Cumberland Plateau (67.5), and LordFairfax (82.7) located in the SouthWest had the lowest. It's also to note that high incidence areas are mostly populated by AA men located in the south-eastern part of the State where they once served as an abundant and cheap workforce for local industries. A spatial analysis of distribution per race with stratification by income and education levels was not statistically correlated with the racial disparities[292]. Here again, like in the Caribbean Islands, the same combination of *dramatic environmental stressors* including suppression and environmental toxicant is at play. Kepone (decachlorooctahydro-1,3,4 – metheno-2H-cyclobuta [cd] pentalen-2-one) was manufactured in the 1970s in Hopewell, VA. The pesticide was mostly exported in Central and South America to eradicate banana root borer[293]. By late 1975, the EPA evidenced health effects on the workers at the factory and further investigation identified the pollution of the James River by the product. Production was halted and a 9-year fishing ban was imposed on the river and its eastern affluents. Interestingly, the areas considered as the most susceptible to have been contaminated (Fig.17) by the environmental toxicants are the areas recording the highest incidence of prostate cancer (Fig.18).

Figure 17: The building of the Dramatic Environmental Stressors :
African American Cotton Farmers in the US (1890) From Wikipedia, the Free Encyclopedia

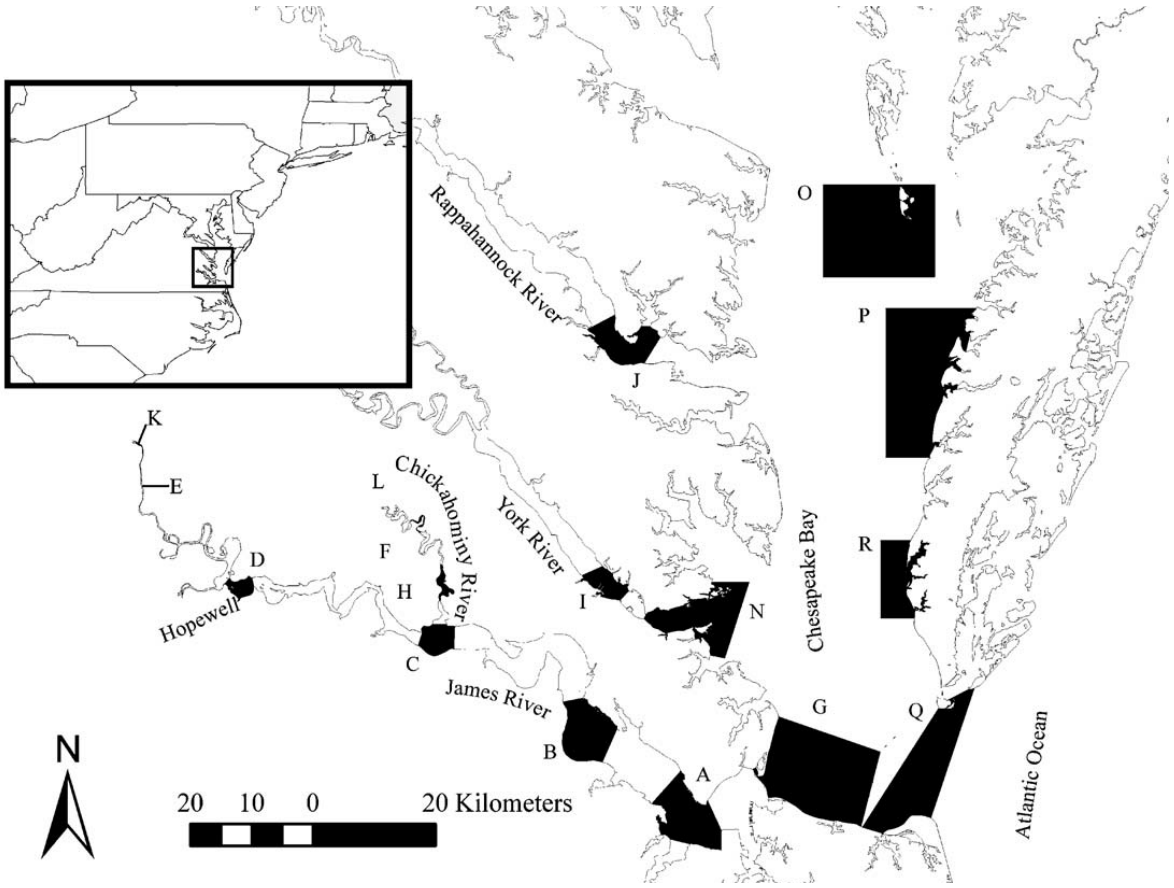

Figure 18: Areas contaminated by Kepone in 1974 in Virginia

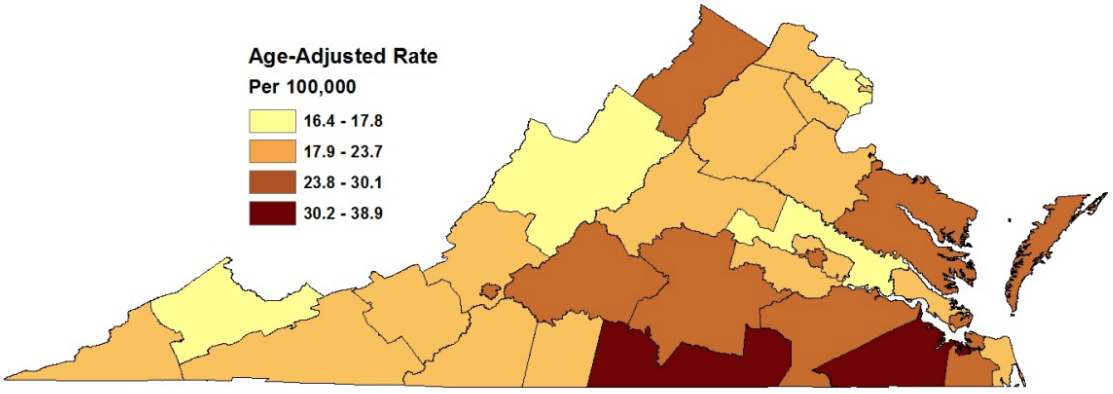

Figure 19: Prostate Cancer incidence in Virginia in 2004.

6. Study Limitations

The study aimed at examining factors that support the incidence patterns of prostate cancer at the populational level, and suggesting a model connecting environmental factors and their biological responses. Nonetheless some limitations can be mentioned.

6.1. Study design

Phase II focused on dietary calcium under the motive that nutrition was the main source of internal body calcium. While this approach is justified given the intense debate on the role of dairy calcium in Western studies, and also addressed the need to have an African Cohort in the Pooling Project, it could however be oversimplifying. There is a wider range of risk factors, t*he dramatic environmental stressors* as we called them in our model. Current empirical data and epidemiological examples suggest that geography , occupation , lifestyle in general, migration, and socio-economic status. Stated otherwise, the African Prostate Cancer Study (APCS), instead of focusing on nutrition only could have also included another study arm focussing on other dramatic environmental stressors such retired farmers. Ivory Coast is a country producing close to 40% of the world cocoa and ranking third for coffee production and other industrial crops. There is poor to none regulation about environmental toxicants used as pesticides and insecticides. Also the country has been under the colonial yoke officially from 1893 to 1960. The context seems then quite similar to our case studies it would therefore be quite informative for the APCS to investigate beyond nutrition exposure to other *dramatic environmental stressors*.

Also, Phase III of the research when it's completed can be difficult to interpret in regard to the general theory developed here. According to our model, epigenetic pathways have precedence on genetic pathways. Thus the African American cell lines (hT E006AA) and the European American cell line (LNCaP) should respond identically to the Peptide#1 stimulus. What if the response is different ? It then appears that the interest of Peptide#1 is more therapeutic than diagnostic. In the population health paradigm as elaborated by Rose, *treatment* of the most vulnerable populations takes precedence on the *treatment* of the sick individuals[72].

6.2. The Role of Calcium

Although the approach of tumor development from the calcium homeostasis angle is based on rigorous experimental findings as well as on some logical inferences and on a coherent systemic overview, some limitations to the theory should be acknowledged. The first one is the capability to evidence the causal calcium wave during the two phases (increase and habituation) described earlier. Jaffe suggested a decade ago an experiment based on aequoerinated mice which will allow the follow up of the calcium signal from its induction to the different impacts on cell structures and compartments[139]. Another challenge is the technical capabilities to measure local variations in calcium concentrations such as at the matrix level and in the different cell compartments, calcium trafficking and concentrations being the core of the theory. However, the most challenging remains the universality of calcium signal in biological systems. It is generally opposed that calcium is too universal a cell signal to make a good target against cancer. If the argument has some weight, it should nevertheless be balanced by the versatility of the calcium signal, as suggested by Berridge [82]. In other words, each tissue has, in theory, its own specificity in regard to calcium homeostasis, as it has been developed throughout this monograph.

Chapter 8: Conclusion and Next Steps

Our research describe the design, method and preliminary findings on the ethnoracial disparities of prostate cancer. The study is conducted in three different phases, Phase I is a critical and analytical review of empirical studies and experiments that have been conducted on the biological role of calcium in malignancies and how it applies to prostate tumorigenesis. That work was published in CMR in 2016. Phase II of the study aimed at investigating one risk factor of prostate cancer in a large population study. The African Prostate Cancer Study (APCS) protocol and pilot study is currently under review. As mentioned earlier this suggested model of cancer disparities was presented as the 10th Annual Conference of the American Association for Cancer Research in September 2017. At this point in our multi-method research some findings can be outlined.

1. Prostate Cancer incidence and mortality in men from African ancestry

The two segments of prostate cancer disparities have two distinct explications :
(*i*) a disparity in incidence. This could be explained by the historic changes that have resulted in the differentiated exposure of men of African Ancestry to *dramatic environmental stressors* (calcium intake patterns or diet, social stress, environmental toxicants, less exposure to sunlight, and new exposure to colder temperatures) and the *survival* biological response triggered. These responses involved primarily the neuroendocrine system and were mediated by calcium cellular metabolism, then epigenetically and transgenerationally transferred from parents to their offsprings. As a result, an in increase in proliferative activity of glandular tissues such as the prostate observed in the descendants. (*ii*) a disparity in outcome. This is mediated calcium: a historically impaired calcium signal in the neuroendocrine tissues tends to promote a faster proliferation, in order to augment *survival* combined with socio-economic factors such as financial barrier, trust, cultural perceptions, affecting care seeking behaviors and subsequent delay to treatment. Based on these considerations we suggested two models : an *incidence model* and an *outcome models.* The components of the models are the same : *social factors* and *biological factors* mediated by *calcium*. But the way they interact are different. In the Incidence Model (fig.14), the social factors, because of their suppressive nature, triggers survival biological responses through calcium cellular metabolism, either directly in factors like diets, exposure to sunlight or indirectly in factors like stress or environmental toxicants. Calcium cellular metabolism is in that perspective the way whereby social

inequalities get *under the skin*. In the Outcome Model (fig.15), biological factors and social factors work separately the first precipitating the disease and the second delaying access to treatment. The aim of having causal models in the science of Population Health is to indicate leverage points where action can be taken to curve the risk distribution of the population. What points could be selected as essential for population health intervention?

2. Leverage Points in the Models

Two leverages points can be identified to curb prostate cancer incidence at least in theory. First reduce exposure to the *dramatic environmental stressors.* It should be clarified that they work synergistically therefore focussing on just diet and physical activity as it has been advocated in the recent years by health promotionists [73] is necessary but sufficient to tackle incidence. It should be accompanied by social measures in order to reduce social stress, increase optimal exposure to sunlight. However this factors were more active for the parental generation the extent to which the offsprings could benefit from preventive interventions is more nuanced since the mechanism is epigenetically supported. Nonetheless, if the *dramatic environmental stressors* are progressively contained one can expect that the bodies of the following generations, say *Generation F(n+1)*, will gradually remove the survival mechanisms and therefore correct calcium signal in the neuroendocrine system. Another leverage point reside in treatment. Incidence loses its pertinence if timely and appropriate care exist. From our models, it appears that epigenetic treatments targeting specific calcium signal might prove to be the best options for a cure. Theses measure also have the potential benefit to improve outcomes.

3. Calcium the *environmental messenger*

In the early days of the calcium signaling saga, the divalent cation was established as a first messenger[90] for cellular signal transduction. Then, further work established that not only it was a first messenger, but it could also play as a a *second messenger* as well which implies serving as an intermediary[148]. It is even shown that the cation could served as a third messenger in some molecular processes[294]. In our study we pose a new function to calcium as the messenger of environmental signals: hence the concept of *environmental messenger.* Cancer studies on migrations[295] have established that when individuals migrate from a low incidence cancer area to a high after a few generations the incidence levels of cancer in the offsprings align with those at

destination. Based on the model established for prostate cancer in men of African Ancestry such effect on migrants could be understood as deriving from the *dramatic environmental stressors* and are mediated by calcium, the *environmental messenger*.

4. Future results and research

Two other important results are expected for our study: what could be the correlation between calcium intake and prostate cancer risk in subsaharan Africa ? And how will prostate cell lines respond to a synthetic calcium binding peptides ? Those results will help refined to the theory developed in this monograph. The APCS should confirm that changes in calcium intake patterns similar to what the ancestor of the African American faced when they were deported is sufficient to trigger the disease or it's a conjunction of all the factors. The APCS Food Frequency Questionnaire validated in the pilot study, is designed to capture not only nutrition but some social factors as well. The proteomic study should serve as a confirmation at the molecular level and open the possibility for a an epigenetic drugs through Peptide #1. However, the charm of Population Health beyond its multidisciplinarity, is that a model for one disease has the potential to work for a group of diseases. As mentioned earlier, it's the whole neuroendocrine system that is involved in the biological mechanisms. Therefore, groups of diseases like high blood pressure which is, unsurprisingly, more frequent in African American males than any other population, could be tackled using the same model.

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Appendix

Appendix 1 : The African Prostate Cancer Study Food Frequency Questionnaire (APCS-FFQ)

Appendix 2 : Ethic Approval N° : H03-17-20

ETUDE DU REGIME ALIMENTAIRE CHEZ LES RETRAITES IVOIRIENS

Note :

1. Ce questionnaire peut être rempli seul ou avec assistance. Il permet de calculer de façon approximative la quantité de calcium dans l'alimentation. Son remplissage est libre, volontaire et anonyme il est à but exclusivement scientifique et dure environ 30mn.

2. En cas de question, prière contacter :
   a. Par téléphone :……
   b. Par e-mail :…….

Fiche d'enquête # /___/___/___/

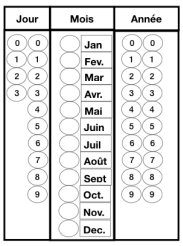
Enquêteur (trice) # /___/___/

Date de l'enquête /____/____/_____/

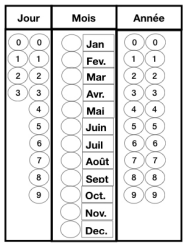
Numéro de Dossier (Ne pas remplir) ☐☐☐☐

Nom ☐☐☐☐☐☐☐☐☐ Prénom ☐☐☐☐☐☐☐☐☐ Ville ☐☐☐☐☐☐☐☐☐

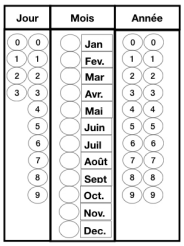
1.Date de Naissance



2. Année de Retraite



3. Date d'aujourd'hui



5. District d'habitation depuis la retraite :

Abidjan Métro (District 1)	Montagnes (District 2)	Sassandra marahoué (District 3)	Bas-Sassandra (District 4)	Goh-Djiboua (District 5)	Savanes (District 6)	Lagunes (District 7)	Vallée du Bandama (District 8)	Lacs (District 9)	Comoé (District 10	Zanzan (District 11)	Moroba (District 12)	Denguélé (District 13)	Yakro (District 14)
○	○	○	○	○	○	○	○	○	○	○	○	○	○

6. Vous habitez en zon

Urbaine	○
Rurale	○

7. Numéro de téléphone : /_____/

8. Numéro de téléphone d'un proche /_____/

8. Profession antérieure : ……

9.Poids :…… 10. Taille…… (cm) 11.Tension artérielle……12.Glycémie ……..

13.Pratiquez-vous le Sport ? ……14. A quelle fréquence ?.....

15. Régime alimentaire : 15.1.Normal, 15.2 Sans sel 15.3.Sans sucre 15.4.Végétarien

16. Prenez-vous des compléments nutritionnels ? 16.1. Oui 16.2. Non

16.3 Si oui veuillez préciser lesquels ……

17. Avez-vous une (des) maladie(s) pour la(les)quelle(s) vous êtes médicalement suivis ?
17.1 Oui 17.2 Non

17.3 Si oui veuillez préciser la(les)quelle(s) ……

18. Fumez-vous de la cigarette ? 18.1. Oui 18.2. Non

18.3 Si oui veuillez préciser combien de cigarettes/jour ……

19. Consommez-vous de l'alcool ? 18.1. Oui 18.2. Non

***Examen Prostatique* :**

Date : ……

Avez-vous récemment eu des troubles urinaires ? ……

TR : ……

PSA : ……

Les Produits Laitiers	***Combien de fois ?***								***En quelle quantité?***
	Rarement ou Jamais	1 à 3 fois /M	1 fois/S	2 à 4 fois/S	5 à 6 fois/S	1fois/ jour	2 à 3 fois /J	Plus de 4 fois/J	
Lait entier naturel	○	○	○	○	○	○	○	○	○ 1 verre ○ 2 verres ○ 3 verres ○ > 3 Verres
Lait demi-écrémé	○	○	○	○	○	○	○	○	○ 1 verre ○ 2 verres ○ 3 verres ○ > 3 Verres
Lait écrémé	○	○	○	○	○	○	○	○	○ 1 verre ○ 2 verres ○ 3 verres ○ > 3 Verres
Lait concentré *sucré.*	○	○	○	○	○	○	○	○	○ 1 verre ○ 2 verres ○ 3 verres ○ > 3 Verres
Lait concentré *non-sucré.*	○	○	○	○	○	○	○	○	○ 1 verre ○ 2 verres ○ 3 verres ○ > 3 Verres
Lait en poudre *entier.*	○	○	○	○	○	○	○	○	○ 1 verre ○ 2 verres ○ 3 verres ○ > 3 Verres
Lait en poudre *écrémé*	○	○	○	○	○	○	○	○	○ 1 verre ○ 2 verres ○ 3 verres ○ > 3 Verres
Lait en poudre *demi-écrémé.*	○	○	○	○	○	○	○	○	○ 1 verre ○ 2 verres ○ 3 verres ○ > 3 Verres
Lait entier naturel	○	○	○	○	○	○	○	○	○ 1 verre ○ 2 verres ○ 3 verres ○ > 3 Verres
Gâteaux	○	○	○	○	○	○	○	○	○ 1 verre ○ 2 verres ○ 3 verres ○ > 3 Verres

Les Produits Laitiers	***Combien de fois ?***								***En quelle quantité?***
	Rarement ou Jamais	1 à 3 fois /M	1 fois/S	2 à 4 fois/S	5 à 6 fois/S	1fois/ jour	2 à 3 fois /J	Plus de 4 fois/J	
Yaourt nature non sucré	○	○	○	○	○	○	○	○	○ 1 pot ○ 2 pots ○ 3 pots ○ > 3 pots
Yaourt nature sucré	○	○	○	○	○	○	○	○	○ 1 pot ○ 2 pots ○ 3 pots ○ > 3 pots
Céréales au lait (riz, mil…) Dêguê.	○	○	○	○	○	○	○	○	○ 1 verre ○ 2 verres ○ 3 verres ○ > 3 Verres
Café au lait	○	○	○	○	○	○	○	○	○ 1 Tasse ○ 2 Tasses ○ 3 Tasses ○ > 3 Tasses
Yaourt	○	○	○	○	○	○	○	○	○ 1 pot ○ 2 pots ○ 3 pots ○ > 3 pots
Boissons lactées	○	○	○	○	○	○	○	○	○ 1 verre ○ 2 verres ○ 3 verres ○ > 3 Verres
Fromage *La vache qui rit*	○	○	○	○	○	○	○	○	○ 1 portion ○ 2 portions ○ 3 portions ○ > 3 portions
Fromage *Kiri*	○	○	○	○	○	○	○	○	○ 1 portion ○ 2 portions ○ 3 portions ○ > 3 portions
Fromage *Brie de Maux*	○	○	○	○	○	○	○	○	○ 1 portion ○ 2 portions ○ 3 portions ○ > 3 portions
Fromage *Gruyère*	○	○	○	○	○	○	○	○	○ 1 portion ○ 2 portions ○ 3 portions ○ > 3 portions
Fromage *Camembert*	○	○	○	○	○	○	○	○	○ 1 portion ○ 2 portions ○ 3 portions ○ > 3 portions
Fromage *Parmesan*	○	○	○	○	○	○	○	○	○ 1 portion ○ 2 portions ○ 3 portions ○ > 3 portions
Croissant	○	○	○	○	○	○	○	○	○ 1 portion ○ 2 portions ○ 3 portions ○ > 3 portions

Les Produits Laitiers	***Combien de fois ?***								***En quelle quantité?***
	Rarement ou Jamais	1 à 3 fois /M	1 fois/S	2 à 4 fois/S	5 à 6 fois/S	1fois/ jour	2 à 3 fois /J	Plus de 4 fois/J	
Chocolat en barre (Lindt,…)	○	○	○	○	○	○	○	○	○ 1 barre ○ 2 barres ○ 3 barres ○ > 3 barres
Chocolat en poudre (Milo, Nescao, Nesquick…)	○	○	○	○	○	○	○	○	○ 1 sachet ○ 2 sachets ○ 3 sachets ○ > 3 sachets
Chocolat en pate (Nutella, Chocomax,…)	○	○	○	○	○	○	○	○	○ 1 cuillèrée ○ 2 cuillèrées ○ 3 cuillèrées ○ > 3 cuillèrées
Beurres Plaquettes 25g	○	○	○	○	○	○	○	○	○ 1plaquette ○ 2plaquettes ○ 3 plaquettes ○ > 3 plaquettes
Glaces	○	○	○	○	○	○	○	○	○ 1 boules ○ 2 boules ○ 3 boules ○ > 3 boules
Pizza	○	○	○	○	○	○	○	○	○ 1 portion ○ 2 portions ○ 3 portions ○ > 3 portions
Lasagne	○	○	○	○	○	○	○	○	○ 1 portion ○ 2 portions ○ 3 portions ○ > 3 portions
Gâteau	○	○	○	○	○	○	○	○	○ 1 portion ○ 2 portions ○ 3 portions ○ > 3 portions

Poissons	*Combien de fois ?*								
	Rarement ou Jamais	1 à 3 fois /M	1 fois/S	2 à 4 fois/S	5 à 6 fois/S	1fois/ jour	2 à 3 fois /J	Plus de 4 fois/J	***En quelle quantité?***
Poisson séché en sauce	○	○	○	○	○	○	○	○	○ Entier ○ 3 Morceaux ○ 2 Morceau ○ 1 Morceau
Poisson fumé en sauce	○	○	○	○	○	○	○	○	○ Entier ○ 3 Morceaux ○ 2 Morceau ○ 1 Morceau
Poisson braisé	○	○	○	○	○	○	○	○	○ Entier ○ 3 Morceaux ○ 2 Morceau ○ 1 Morceau
Poisson frais en sauce	○	○	○	○	○	○	○	○	○ Entier ○ 3 Morceaux ○ 2 Morceau ○ 1 Morceau
Poisson frit en sauce	○	○	○	○	○	○	○	○	○ Entier ○ 3 Morceaux ○ 2 Morceau ○ 1 Morceau
Poisson séché avec attiéké ou ragoût	○	○	○	○	○	○	○	○	○ Entier ○ 3 Morceaux ○ 2 Morceau ○ 1 Morceau
Poisson fumé avec attiéké ou ragoût	○	○	○	○	○	○	○	○	○ Entier ○ 3 Morceaux ○ 2 Morceau ○ 1 Morceau
Poisson frit avec attiéké ou ragoût	○	○	○	○	○	○	○	○	○ Entier ○ 3 Morceaux ○ 2 Morceau ○ 1 Morceau
Poisson en conserve (Sardines…)	○	○	○	○	○	○	○	○	○ Entier ○ 3 Morceaux ○ 2 Morceau ○ 1 Morceau

20. Quels types de poisson consommez-vous le plus ? Numérotez-les par ordre du plus fréquent au moins fréquent

Tilapia ☐
Sardine ☐
Machoiron ☐
Hareng ☐
St Pierre ☐
Raie ☐
Thon ☐
Maquereau ☐
Sole ☐
Merlan ☐
Truite ☐
Morue ☐
Capitaine ☐
Carpe ☐
Silure ☐
Petits poissons de mer séchés ☐

Boeuf , mouton cabri	*Combien de fois ?*								*En quelle quantité?*
	Rarement ou Jamais	1 à 3 fois /M	1 fois/S	2 à 4 fois/S	5 à 6 fois/S	1fois/ jour	2 à 3 fois /J	Plus de 4 fois/J	
Viande de bœuf séchée en sauce	○	○	○	○	○	○	○	○	○ Entier ○ 3 Morceaux ○ 2 Morceau ○ 1 Morceau
Viande de bœuf fumé en sauce	○	○	○	○	○	○	○	○	○ Entier ○ 3 Morceaux ○ 2 Morceau ○ 1 Morceau
Viande de boeuf fraiche en sauce	○	○	○	○	○	○	○	○	○ Entier ○ 3 Morceaux ○ 2 Morceau ○ 1 Morceau
Viande de bœuf grillée en sauce	○	○	○	○	○	○	○	○	○ Entier ○ 3 Morceaux ○ 2 Morceau ○ 1 Morceau
Viande bœuf braisée (Choukouya)	○	○	○	○	○	○	○	○	○ Entier ○ 3 Morceaux ○ 2 Morceau ○ 1 Morceau
Viande de mouton fumé	○	○	○	○	○	○	○	○	○ Entier ○ 3 Morceaux ○ 2 Morceau ○ 1 Morceau
Viande de mouton braisée (choukouya)	○	○	○	○	○	○	○	○	○ Entier ○ 3 Morceaux ○ 2 Morceau ○ 1 Morceau
Viande dem mouton en sauce	○	○	○	○	○	○	○	○	○ Entier ○ 3 Morceaux ○ 2 Morceau ○ 1 Morceau
Viande de cabri en sauce	○	○	○	○	○	○	○	○	○ Entier ○ 3 Morceaux ○ 2 Morceau ○ 1 Morceau
Viande de cabri braisée	○	○	○	○	○	○	○	○	○ Entier ○ 3 Morceaux ○ 2 Morceau ○ 1 Morceau
Viande de cabri fumée	○	○	○	○	○	○	○	○	○ Entier ○ 3 Morceaux ○ 2 Morceau ○ 1 Morceau

Poulet et viande	*Combien de fois ?*								
	Rarement ou Jamais	1 à 3 fois /M	1 fois/S	2 à 4 fois/S	5 à 6 fois/S	1fois/ jour	2 à 3 fois /J	Plus de 4 fois/J	***En quelle quantité?***
Poulet en sauce	○	○	○	○	○	○	○	○	○ Entier ○ 3 Morceaux ○ 2 Morceau ○ 1 Morceau
Poulet braisé	○	○	○	○	○	○	○	○	○ Entier ○ 3 Morceaux ○ 2 Morceau ○ 1 Morceau
Œuf bouilli ou omelette									○ Plus de 3 ○ 3 œuf ○ 2 œufs ○ 1 œuf
Poulet fumé	○	○	○	○	○	○	○	○	○ Entier ○ 3 Morceaux ○ 2 Morceau ○ 1 Morceau
Viande de brousse en sauce	○	○	○	○	○	○	○	○	○ Entier ○ 3 Morceaux ○ 2 Morceau ○ 1 Morceau
Viande de brousse fumée	○	○	○	○	○	○	○	○	○ Entier ○ 3 Morceaux ○ 2 Morceau ○ 1 Morceau

Crabes et fruits de mer	*Combien de fois ?*								
	Rarement ou Jamais	1 à 3 fois /M	1 fois/S	2 à 4 fois/S	5 à 6 fois/S	1fois/ jour	2 à 3 fois /J	Plus de 4 fois/J	***En quelle quantité?***
Crabe en sauces	○	○	○	○	○	○	○	○	○ Entier ○ 3 Morceaux ○ 2 Morceau ○ 1 Morceau
Fruits de mer	○	○	○	○	○	○	○	○	○ Entier ○ 3 Morceaux ○ 2 Morceau ○ 1 Morceau
Escargot séché	○	○	○	○	○	○	○	○	○ Entier ○ 3 Morceaux ○ 2 Morceau ○ 1 Morceau
Escargot braisé	○	○	○	○	○	○	○	○	○ Entier ○ 3 Morceaux ○ 2 Morceau ○ 1 Morceau
Escargot fumé	○	○	○	○	○	○	○	○	○ Entier ○ 3 Morceaux ○ 2 Morceau ○ 1 Morceau

Accompagnement	*Combien de fois ?*								
	Rarement ou Jamais	1 à 3 fois /M	1 fois/S	2 à 4 fois/S	5 à 6 fois/S	1fois/ jour	2 à 3 fois /J	Plus de 4 fois/J	*En quelle quantité?*
Foutou banane	○	○	○	○	○	○	○	○	○ > 4 plats ○ 3 Plats ○ 2 Plats ○ 1 Plat
Foutou Igname	○	○	○	○	○	○	○	○	○ > 4 plats ○ 3 Plats ○ 2 Plats ○ 1 Plat
Attiéké									○ > 4 plats ○ 3 Plats ○ 2 Plats ○ 1 Plat
Manioc	○	○	○	○	○	○	○	○	○ > 4 plats ○ 3 Plats ○ 2 Plats ○ 1 Plat
Foufou banane	○	○	○	○	○	○	○	○	○ > 4 plats ○ 3 Plats ○ 2 Plats ○ 1 Plat
Foufou igname	○	○	○	○	○	○	○	○	○ > 4 plats ○ 3 Plats ○ 2 Plats ○ 1 Plat
Riz	○	○	○	○	○	○	○	○	○ > 4 plats ○ 3 Plats ○ 2 Plats ○ 1 Plat
Mil	○	○	○	○	○	○	○	○	○ > 4 plats ○ 3 Plats ○ 2 Plats ○ 1 Plat
Sorgho	○	○	○	○	○	○	○	○	○ > 4 plats ○ 3 Plats ○ 2 Plats ○ 1 Plat
Tarro	○	○	○	○	○	○	○	○	○ > 4 plats ○ 3 Plats ○ 2 Plats ○ 1 Plat
Couscous	○	○	○	○	○	○	○	○	○ > 4 plats ○ 3 Plats ○ 2 Plats ○ 1 Plat
Pain	○	○	○	○	○	○	○	○	○ > 4 plats ○ 3 Plats ○ 2 Plats ○ 1 Plat

Sauces	*Combien de fois ?*								
	Rarement ou Jamais	1 à 3 fois /M	1 fois/S	2 à 4 fois/S	5 à 6 fois/S	1fois/ jour	2 à 3 fois /J	Plus de 4 fois/J	*En quelle quantité?*
Gombo Sec	○	○	○	○	○	○	○	○	○ > 4 plats ○ 3 Plats ○ 2 Plats ○ 1 Plat
Gombo Frais	○	○	○	○	○	○	○	○	○ > 4 plats ○ 3 Plats ○ 2 Plats ○ 1 Plat
Feuille de baobab									○ > 4 plats ○ 3 Plats ○ 2 Plats ○ 1 Plat
Sauce kplala	○	○	○	○	○	○	○	○	○ > 4 plats ○ 3 Plats ○ 2 Plats ○ 1 Plat
Sauce aubergine	○	○	○	○	○	○	○	○	○ > 4 plats ○ 3 Plats ○ 2 Plats ○ 1 Plat
Sauce graine	○	○	○	○	○	○	○	○	○ > 4 plats ○ 3 Plats ○ 2 Plats ○ 1 Plat
Sauce arachide	○	○	○	○	○	○	○	○	○ > 4 plats ○ 3 Plats ○ 2 Plats ○ 1 Plat
Sauce légume (Tomate)	○	○	○	○	○	○	○	○	○ > 4 plats ○ 3 Plats ○ 2 Plats ○ 1 Plat
Sauce Feuille de taro	○	○	○	○	○	○	○	○	○ > 4 plats ○ 3 Plats ○ 2 Plats ○ 1 Plat
Sauce Feuille dah	○	○	○	○	○	○	○	○	○ > 4 plats ○ 3 Plats ○ 2 Plats ○ 1 Plat
Sauce Feuille Epinard	○	○	○	○	○	○	○	○	○ > 4 plats ○ 3 Plats ○ 2 Plats ○ 1 Plat
Sauce Feuille Epinard	○	○	○	○	○	○	○	○	○ > 4 plats ○ 3 Plats ○ 2 Plats ○ 1 Plat
Sauce N'tro	○	○	○	○	○	○	○	○	○ > 4 plats ○ 3 Plats ○ 2 Plats ○ 1 Plat

Spécialités	*Combien de fois ?*								
	Rarement ou Jamais	1 à 3 fois /M	1 fois/S	2 à 4 fois/S	5 à 6 fois/S	1fois/ jour	2 à 3 fois /J	Plus de 4 fois/J	*En quelle quantité?*
Tchep	○	○	○	○	○	○	○	○	○ > 4 plats ○ 3 Plats ○ 2 Plats ○ 1 Plat
Ragoût	○	○	○	○	○	○	○	○	○ > 4 plats ○ 3 Plats ○ 2 Plats ○ 1 Plat
Alloco poisson									○ > 4 plats ○ 3 Plats ○ 2 Plats ○ 1 Plat
Frites	○	○	○	○	○	○	○	○	○ > 4 plats ○ 3 Plats ○ 2 Plats ○ 1 Plat
Schawarma	○	○	○	○	○	○	○	○	○ > 4 plats ○ 3 Plats ○ 2 Plats ○ 1 Plat
Pizza	○	○	○	○	○	○	○	○	○ > 4 plats ○ 3 Plats ○ 2 Plats ○ 1 Plat
Ttiéké Poisson (Garba)	○	○	○	○	○	○	○	○	○ > 4 plats ○ 3 Plats ○ 2 Plats ○ 1 Plat

Université d'Ottawa University of Ottawa
Bureau d'éthique et d'intégrité de la recherche Office of Research Ethics and Integrity

Ethics Approval Notice

Health Sciences and Science REB

Principal Investigator / Supervisor / Co-investigator(s) / Student(s)

First Name	Last Name	Affiliation	Role
Sanni	Yaya	Social Sciences / Others	Supervisor
Bernard	Kadio	Health Sciences / Others	Student Researcher

File Number: H03-17-20

Type of Project: PhD Thesis

Title: A populational and molecular investigation of prostate cancer ethno-racial disparities

Renewal Date (mm/dd/yyyy)	Expiry Date (mm/dd/yyyy)	Approval Type
05/24/2019	05/23/2020	Renewal

Special Conditions / Comments:
N/A

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File Number: H03-17-20 **Date (mm/dd/yyyy):** 05/14/2019

Université d'Ottawa University of Ottawa
Bureau d'éthique et d'intégrité de la recherche Office of Research Ethics and Integrity

This is to confirm that the University of Ottawa Research Ethics Board identified above, which operates in accordance with the Tri-Council Policy Statement (2010) and other applicable laws and regulations in Ontario, has examined and approved the ethics application for the above named research project. Ethics approval is valid for the period indicated above and subject to the conditions listed in the section entitled "Special Conditions / Comments".

During the course of the project, the protocol may not be modified without prior written approval from the REB except when necessary to remove participants from immediate endangerment or when the modification(s) pertain to only administrative or logistical components of the project (e.g., change of telephone number). Investigators must also promptly alert the REB of any changes which increase the risk to participant(s), any changes which considerably affect the conduct of the project, all unanticipated and harmful events that occur, and new information that may negatively affect the conduct of the project and safety of the participant(s). Modifications to the project, including consent and recruitment documentation, should be submitted to the Ethics Office for approval using the "Modification to research project" form available at: https://research.uottawa.ca/ethics/forms.

Please submit an annual report to the Ethics Office four weeks before the above-referenced expiry date to request a renewal of this ethics approval. To close the file, a final report must be submitted. These documents can be found at: https://research.uottawa.ca/ethics/forms.

If you have any questions, please do not hesitate to contact the Ethics Office at extension 5387 or by e-mail at: ethics@uOttawa.ca.

[redacted]

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