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ACADEMIC RESEARCH

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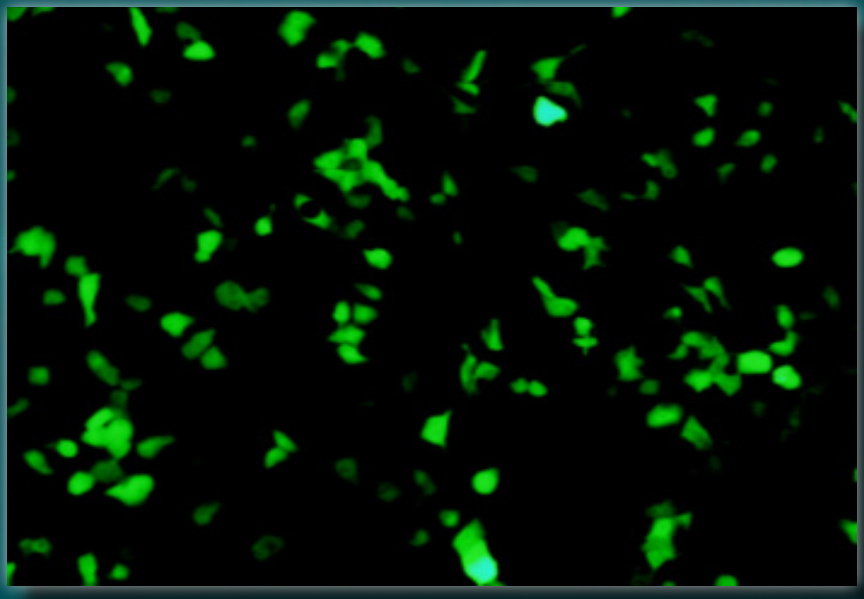
H19 LncRNA and cancer axis of evil: Insights into deadly signature

Pages 9-15



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Al-Quds Journal for Scientific Research

Front Page Figure

Bladder cancer cells express GFP reflect high activity of the H19 gene promoter.

Al-Quds Journal for Scientific Research

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Aims And Scope

Al-Quds Journal for Academic Research is a peer reviewed multidisciplinary journal covering wide areas of research in the fields of natural sciences, Medical and Biological sciences, Humanities, Arts and Social sciences. The journal publishes reviews in hot fields of research. The journal issued on biannual basis; on January the issue will cover research in natural, medical and biological sciences and on June the issue will publish research in Humanities and Arts. Al-Quds Journal for Academic Research accepts research in both English and Arabic Languages for arts and humanities fields and only in English for natural, medical and biological sciences.

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Founded in 1984, is a Palestinian university, and the only university in the world to be situated by a separation wall. The main campus of the University is located in Abu Dis with four further campuses in Jerusalem, Sheikh Jarrah, Beit Hanina and Rammalh (al-Bireh). AQU provides higher education and community services within the Jerusalem area as well as the neighboring towns, villages and refugee camps in the West Bank. With over 13,000 students, AQU is wide reaching with members from all over the West Bank, including from the districts of Bethlehem, Hebron, Jenin, Jericho, Nablus, Ramallah, Tulkarem, and Qalqilia. With fifteen academic faculties including Arts, Medicine, Dentistry, Law, Qur'an and Islamic Studies and Engineering, AQU has huge academic diversity and offers something for everyone. AQU is a hub of creativity and offers a vibrant learning environment by encouraging an exchange of ideas and freedom of expression, as well as leading innovative research. This allows for the highest standard of education for our students, as well as encouraging them to be active engaged citizens, who are open and cooperative, as well as well informed and interested in world cultures. In order to stay at the forefront of academia, AQU has international partnerships with American and European universities. It is also affiliated with over 30 institutes and engaged in innovative research around the world.

Al-Quds Journal for
Academic Research

The Plastic Industry worldwide and in Palestine

OPENION

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A world without plastics or synthetic polymers can't be imagined today. The first synthetic plastics was produced in the beginning of the twentieth century, however industrial plastics production started in 1950. Production of plastic materials to day surpasses any other synthetic material with the exception of steel and cement. The share of plastics in municipal solid waste increased from 1% in the 1960 to more than 10% in 2005. Most monomers used today to make plastics such polyethylene (PE) or Polypropylene (PP), or polystyrene (PS) are produced from the petroleum industry and none is biodegradable, they accumulate in the environment and pose great threat and serious concern to humanity and to marine life.

In 2010 approximately 8 Million Metric Ton (MT) of plastic waste entered the marine environment. Global production of polymers and fiber increased from 2 (MT) in 1960 to 380(MT) in 2015 a compound annual growth rate (CAGR) of 8.4% while the total production of polymers and fibers from 1960 – 2015 was estimated to be around 7800 (MT). China alone produces 28%, and 68% of world production of PP. Biodegradable plastics amount to only 4 (MT). Non fiber plastics production is (PE 36%, PP 21%), Polyvinylchloride PVC (12%) followed by polyethylene terphthalate PET, polyurethane, and polystyrene less than 10% each ,42% of plastics are used in packaging.

Palestine show a fast-growing plastic industry though we import plastics worth 255 million US $ as reported in the United Nations International Trade Statistics (COMTRADE) in 2018, compared to US $200 Million imported in 2014. However, we were able to export to the world 66.3 million US $ worth of plastic materials added to that our export to Israel of plastic product worth 86 million US $, mostly packaging materials.Three important countries that export plastic materials to Palestine are Turkey. China and south Korea. Turkey alone in 2018 exported plastics worth 25 million $.

The plastic industry in Palestine is among the largest industry. However, we still manufacture the traditional plastics for packaging. Our country needs to develop this industry and diversify the plastic products to meet the needs of the market such as automobile, electrical appliances, refrigerators, and many other industries.

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Al-Quds Journal for
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Prodrugs from Serendipity to Design by Computational Chemistry Methods

EDITORIAL

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Imagination is more important than knowledge when knowledge is limited and can not solve important questions. Inventiveness in the drug design has been clumsiness in quality and quantity. This may be due to the ineptness and incapability of medicinal chemists to comprehend biochemistry and biology issues. On the other hand, biochemists, biologists, and pharmaceutical chemists do not possess the expertise to make complex organic entities. Hence, a team comprising of all expertise is a must to invoke a novel drug.

Drug discovery and development is expensive and time-consuming since it consists of many steps that start with target and lead discovery and end with human clinical trials. The estimation is that about 10-15 years are needed to present a new drug to the market with a cost of 1-1.5 billion dollars (Figure 1), (Karaman 2014 a,b). During the recent few decades, considerable attention has been focused on improving the pharmacokinetics of existing marketed drugs, thus providing new organic entities capable of providing more efficiency with fewer drawbacks than their corresponding parent drugs. Among the approaches that can fulfill the requirements for invoking therapeutics with optimum absorption, distribution, metabolism, and excretion (ADME) properties is the prodrug approach.

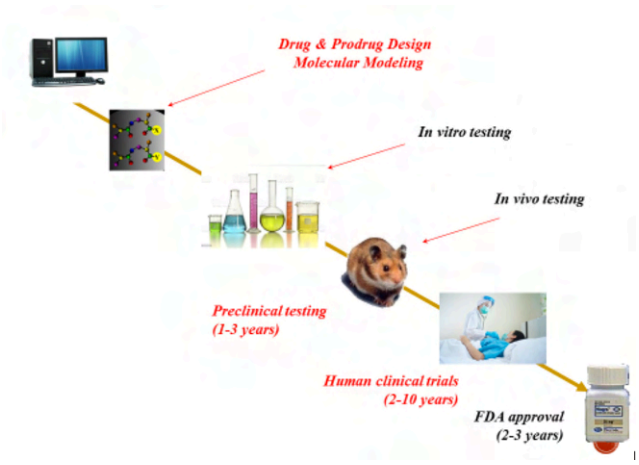

Figure 1. A diagram illustrating the different stages in drug and prodrug development.

This approach is considered a well-established strategy with a high potential of success in obtaining organic moieties having a vast efficacy and negligible toxicity.

It is about a tenth of all worldwide marketed drugs are listed as prodrugs, and in the recent ten years their percentage was significantly increased and their share of all approved small molecular weight medicines reached 30%, and this high percentage signifies the great success of this approach. A prodrug is a chemically modified inactive agent consisting of a non-toxic promoiety and pharmacologically active moiety which upon exposure to a physiologic environment liberates the pharmacologically active parent drug to elicit its therapeutic benefits (Figure 2). Prodrugs are designed to undergo interconversion in physiologic environments via enzyme catalysis

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or chemical reactions. In both cases, the prodrug interconversion rate is not controlled by the designer chemist but by the abundance of certain enzymes in the route of administration. Generally, the yield of interconversion by enzymes is less than 50% and could be varied among persons with different ethnicity

Among the great number of prodrugs exist in the market are the ant rheumatic agent oxindole succinate, the anti-inflammatory drugs valdecoxib, prednisolone, and fluocinolone acetonide, the anti-glaucoma agent dipivefrin, the anticonvulsant agent progabide, the antiviral agent valacyclovir, the analgesic buprenorphine decanoate, fluphenazine decanoate to treat chronic schizophrenia, naltrexone ester prodrugs for narcotic dependence, nalbuphine ester as analgesic, and mestranol for birth control.

The classical prodrug approach by which the intercoversion of the prodrug is achieved via enzyme catalysis has scored a significant success in reducing toxicity and increasing the bioavailability of many drugs. On the other hand, prodrugs designed to release the parent active drug through inter or intramolecular reaction without enzyme catalysis is a more advantageous approach since it lacks the intra-individual variability caused by the metabolic enzymes.

In this editorial, we discuss a modern approach that implies the design of prodrugs that interconvert to their corresponding parent drugs through an intramolecular process. In this approach, molecular orbital and molecular mechanics methods are used to estimate reaction rates which then are correlated with experimental rate values. In this approach, there is no need for an enzyme to catalyze the process, and the cleavage rate of the prodrug to its parent drug is solely determined on the rate-limiting step of the process which is entirely dependent on the nature of the prodrug's promoiety.

For several decades, the extraordinary efficiency of enzymes has been modeled by several well-known chemists and biochemists. Menger, Bruice, Kirby, Bender, and Jencks have assembled enzyme model devices that are capable of obtaining rates similar to that observed with enzyme-catalyzed reactions. Striking examples of such devices are those invoked by Menger, Kirby, and Bruice in which the extraordinary rate acceleration is due to covalently enforced proximity. For more than six decades, computational methods have been utilized by inorganic, organic, organometallic, and pharmaceutical chemists alike for predicting the physical, chemical, and molecular properties of compounds. Molecular orbital methods such as DFT, a semi-empirical and ab initio, and molecular mechanics methods have proven to be successful tools for the prediction of thermodynamic and kinetic parameters for biological moieties that have pharmaceutical / medicinal interest; drugs and prodrugs (Karaman 2011).

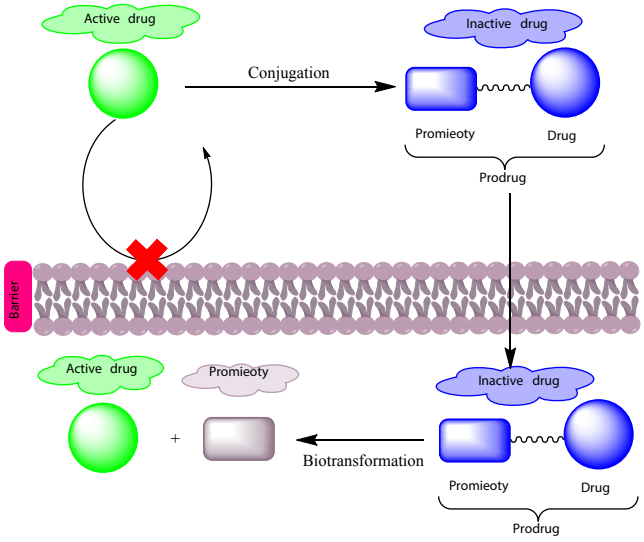

Figure 2: A diagram showing the prodrug concept.

Aiming to design novel prodrugs for commonly used drugs that suffer from reduced bioavailability or/ and bitter sensation we have used DFT and molecular mechanics methods to invoke the mechanisms and assign the factors determining the reaction rates in several intramolecular processes. Among these intramolecular reactions are: (1) cyclization reactions of di-carboxylic semi-esters by Bruice and Pandit, (2) lactonization of hydroxy-acids by Cohen and

Milstein and Menger, (3) proton transfer between two oxygen's in Kirby's acetals and proton transfer between nitrogen and oxygen in Kirby's carboxylic amines, (4) proton transfer between two oxygens in rigid carboxylic amides by Menger et al. (5) proton transfer between two oxygens in N- alkylmaleamic acids by Kirby. The information gained from these studies was utilized to design an efficient chemical entity to be exploited as a prodrug promoiety having the potential to release the parent drug in a programmable manner. For instance, unraveling the proton transfer mechanism of Kirby's acetals has resulted in the design of azanucleosides' prodrugs for the treatment of myelodysplastic syndromes, where the prodrug promoiety is linked to the hydroxyl group of the nucleoside.

Furthermore, paracetamol prodrugs lacking the bitter sensation of the parent drug, paracetamol, have been through linking the hydroxyl phenolic group to a linker, thus inhibiting any biding with the bitter taste receptors. A variety of different linkers were also studied for the design of several prodrugs including the anticonvulsant agent gabapentin, the antihypertensive agent, atenolol, the anti-Parkinson's agent dopamine, the anti-viral agent acyclovir, and the anti-malarial agent, atovaquone, the anti-bleeding agent, tranexamic acid, and the antibacterial agents, amoxicillin, and cephalexin.

This novel approach which is relatively fast and with minimum cost can provide new prodrugs with increased bioavailability, reduced bitter sensation, and enhanced dissolution and membrane penetration (Haddad et al. 2018, Karaman 2014 c).

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The long Non-coding RNA Orchestrator of Cancer Axis of Evil Insights into the Multiple Modes of Action of the H19 Gene

REVIEW

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A B S T R A C T

Increasing evidence has indicated that the non-coding RNA molecules play central roles in almost all biological processes and many pathological conditions including carcinogenesis. This review focuses on the pathological tumorigenic role of the first discovered long non-coding RNA gene called H19 and its pivotal contribution to the cancer axis of evil. H19 RNA utilizes a variety of mechanisms to perform its pathological function. Some key unanswered questions are presented by the end. Understanding the H19 RNA mechanisms of action will shed light into the class of long non-coding RNA which contains contains thousands of members mostly with unknown functionn and will help in delineating the pathological role played by at least some of them

Keywords: Long non-coding RNA, Competing endogenous RNA, Epigenetic regulation, microRNA sponge Epithelial to mesenchymal transition, Cancer stemness, Drug resistant, Exosome

After realizing that our DNA is a transcription machine, with a little protein coding potential, the past decade has witnessed an explosion in scientific researches reporting the identifications, characterizations and exploration of the modes of action of the non-coding RNA (ncRNA) genes. Currently, the ncRNA research field is one of the most popular fields in biological and medical sciences. An ncRNA is an RNA molecule that is not translated into a protein product and is classified according to its size into a long (more than 200 nucleotide) and a short (less than 200 nucleotide) ncRNA. The vast knowledge accumulated during the past decade was sufficient for the scientific community to announce the entry into the fascinating ncRNA era which was and still dominated by the microRNAs (miRNA) researches. The miRNAs are small ncRNA mediating regulation of gene expression and their discovery has changed vastly the way we used to think about gene regulation.

The functions and mechanisms of action of the long non-coding RNAs (lncRNAs) are the least understood aspect of the ncRNA biology. Many thousands of lncRNAs are produced from our genome, yet relatively very few have well documented roles. This review will handle a gene called H19, which transcribes to an lncRNA.. Being the first imprinted lncRNA discovered, our knowledge on H19 is relatively high compared to the others. This is especially reflected by the steep increase in the scientific reports handling the H19 lncRNA functions and its modes of action that occurred in the past few years after decades of relative dormancy. H19 is expressed in the placenta and in the embryonic stage but shut down in most tissues after birth and re-expressed again in almost all cancer types.

The roles of H19 in cancer are diverse and

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touches almost every aspect of the tumorigenesic process. This review focuses into the well-established mechanistic roles of the H19 lncRNA in the epithelial to mesenchymal transition (EMT), cancer stem cells, and drug resistance, collectively called "cancer axis of evil" (Singh and Settleman, 2010), as they are responsible for the tumor metastasis and the failure of the chemotherapeutic drugs. This dictates indeed the cancer deadly signature. EMT is a reversible trans-differentiation process through which the non-motile epithelial cells are converted to motile mesenchymal cells, a step that is essential for tumor invasion and subsequent metastasis. Cancer stem cells are rare cancer cell population within the tumor that have stem-cell like properties and is greatly believed among the scientific community that these cells are responsible for fueling tumor growth, tumor heterogeneity, drug resistance and invasion. Some examples into other physiological and pathological roles for H19 are presented when needed for clarifications. It is astonishing how H19 can employs diverse mechanisms to perform its specific functions. An illustration of three (among others) well documented modes of action for H19 lncRNA is presented.

H19 lncRNA functions as a scavenger through sponging miRNAs.

One of the well-established modes of action through which the H19 lncRNA performs its function is by acting as a competing endogenous RNA (ceRNA) for the purpose of "sponging" appropriate candidate miRNAs. By this H19 relieves the sponged miRNA inhibitory effect on its downstream targets and thus acts as a scavenger.

One of the earliest reports showed a conserved role for H19 in modulation the major let-7 family availability by acting as a molecular sponge. Consequently, H19 affects expression of endogenous Let-7 targets including Dicer and Hmga2 (Kallen et al., 2013), a multifunctional proteins with broad activities. A double negative feedback loop between H19, let-7 miRNA and the pluripotency factor LIN28 has a critical role in the maintenance of breast cancer stem cell properties (Peng et al., 2018). Additional report indicates that oestrogen induction symmetric division in breast cancer stem-like cells is regulated by H19 through antagonizing Let-7c(Wang et al., 2019). We were the first to document that H19 is induced by hypoxic stress through the Hypoxia-inducible factor 1α (HIF1-α) pathway (Matouk et al., 2010). An interesting recent report indicated that H19 is responsible for glycolysis and breast cancer stem cells (BCSC) maintenance. Mechanistically H19 acting as ceRNA sequesters miRNA let-7 miRNA to release HIF1-α, leading to an increase in pyruvate dehydrogenase kinase 1 (PDK1) expression. PDK1 enhances BCSC properties and is correlated with poor overall survival (Peng et al., 2018).

We have provided several evidences for H19 central role in epithelial to mesenchymal transition (EMT) process (Matouk et al., 2014) and suggested that H19 can also act as an orchestrator for the EMT-MET processes (Matouk et al., 2016). Multiple reports have indicated that this can be performed at least in part through the sponging activity of H19 lncRNA. For instance, by derepressing let-7's suppression on its target HMGA2, H19 promotes EMT and metastasis in pancreatic cancer model (Ma et al., 2013). Additionally, in colorectal cancer it was demonstrated that H19 sponges miR-138 and miR-200a that led to the de-repression of their endogenous targets Vimentin, ZEB1, and ZEB2, all are well established marker genes for mesenchymal cells (Liang et al., 2015) Furthermore it was shown that through differentially sponging miR-200b/c and let-7b, H19 mediates breast cancer cell plasticity during EMT and MET processes (Zhou et al., 2017). In glioma, H19 could compete with SOX4 via sponging miR-130a-3p and thus regulating EMT (Hu et al., 2018). H19 sponges miR-29b-3p and relieve the suppression for DNMT3B, which led to EMT and metastasis of bladder cancer (Lv et al., 2017).

By acting as ceRNA, several reports have indicated that H19 confer cancer chemoresistance in various models. H19 sponges miR-194–5p thus confers 5-Fu resistance in colorectal cancer by promoting SIRT1-mediated autophagy (Wang et al., 2018). Additionally, H19 acts as a miRNA-

106b-5p sponge and thus impairs the function of miRNA-106b-5p on its target gene, TDRG1. By this, H19 facilitate cell survival in cisplatin-based chemotherapeutic conditions in seminoma (Wei et al., 2018). Bortezomib resistance in multiple myeloma is also enhanced by H19 by acting as a miRNA sponge to inhibit the expression of miR-29b-3p, enhance MCL-1 transcriptional translation and inhibit apoptosis (Pan et al., 2019). A recent study indicates that H19 confers resistance to gefitinib via miR-148b/dimethylarginine dimethylaminohydrolase-1 (DDAH1) axis in lung adenocarcinoma. Mechanistically, RNA H19 positively regulated DDAH1 expression via sponging miR-148b-3p (Huang et al., 2020).

H19 is a precursor for miR-675-5p and miR-675-3p

Discovered in 2007 miR-675 processed from the first and longest H19 exon (Cai and Cullen. 2007). MiR-675 was reported to target the "retinoplastoma gatekeeper of DNA replication" (Tsang et al., 2010) and the "p53 Guardian of the genome" (Zheng et al., 2019) both were among the best characterized and well established tumor suppressor genes. The inhibitory effect of miR-675 on p53 could have a myriad consequences given hundreds of targets that p53 has the ability to modulate. We were the first to report that p53 suppress H19 induction upon hypoxic stress (Matouk et al,. 2010) and the upregulation of miR-675 in response to hypoxia (Matouk et al,. 2014). So the possibility of feedback loops between H19-miR-675-P53 by which miR-675b elevate the inhibitory effect of p53 on H19 upon hypoxic stress could be the case. Among other well-known tumor suppressor genes targeted by miR-675 is RUNX1 (Zhuang et al., 2014), and PTEN (Lv et al., 2018) though the latter was not tested in the context of tumorigenicity. Thus it is not astonishing that at least part of the pathological role of H19 is mediated by miR-675. MiR-675 targets a myriad of transcripts in a cellular-context-dependent manner involved in proliferation, apoptosis, EMT, invasion, migration, drug resistance, angiogenesis, and cancer stemness. Although some conflicting data have been reported in different research models, the prevailing view is that miR-675 is functioning as an onco-miR in most models. Physiologically, miR-675 is expressed exclusively in the placenta from the gestational time point when placental growth normally ceases. When lacking H19, the placentas continue to grow suggesting that the physiological role of H19 is to limit placental growth through its microRNA tool. Results indicate that miR-675 slows cell proliferation through at least in part targeting insulin like growth factor receptor 1 (Igf1r), the key receptor through which Igf2 signal to promote growth during fetal development (Keniry et al., 2012).

Additional physiological roles for miR-675 have been described in promoting skeletal muscle differentiation and regeneration (Dey et al., 2014) and the regulation of intestinal epithelial barrier (Zou et al., 2016).

H19 lncRNA epigenetically modulate gene expression

Since H19's discovery in 1984, its locus has been used as a dogma to study epigenetic regulation of the imprinted genes where H19 also act in *cis* modulating and fine tuning the imprinting of other genes within the imprinted cluster where it resides. In this section the emerging role of H19 in epigenetic regulation of gene expression in *Trans* is handled which was uncovered in the past few years. H19 lncRNA interacts with transcription-repressors functioning epigenetically and guide them to specific loci. For example H19 binds the methyl-CpG–binding domain protein 1 (MBD1) and recruits it to some of its targets, by doing so H19 enables the maintaining of repressive H3K9me3 histone marks in their loci (Monnier et al., 2013). Enhancer of zeste homolog 2 (EZH2) is a critical component of Polycomb-Repressive Complex 2 (PRC2). EZH2 is responsible for generating histone H3 lysine 27 trimethylation, a modification that always correlates with transcriptionally repressed chromatin. Several reports uncover a "partnership" between H19 and EZH2. Even more dramatic are reports covering that H19 regulate EZH2 expression itself. EZH2 is regulated by H19, through sponging

of miR-630 (Li et al., 2016). Additionally, and through sponging miR-130, it was reported that H19 regulate EZH2 expression (Hong et al. 2018). The functional outputs of H19-EZH2 association have been reported in a number of studies and using different models. Upregulated H19 enhances bladder cancer metastasis by associating with EZH2 and inhibiting E-cadherin expression. The authors showed that this association resulted in directly suppressing *Ecadherin* transcription and indirectly activating the *Wnt* signaling pathway (Luo et al., 2013). In tongue squamous cell carcinoma, it was shown that H19 promotes carcinoma progression through β-catenin / GSK3β/EMT signaling via association with EZH2 (Zhang et al., 2017). Additionally, in esophageal cancer H19 facilitates EMT and metastasis through let-7c/STAT3/EZH2/β-catenin axis (Chen et al., 2019). Furthermore, in glioblastoma cells H19 regulate NKD1 transcription via EZH2-induced H3K27 trimethylation of its promoter resulting in the repression of Nkd1-a negative regulator of *Wnt* pathway (Fazi et al/. 2018). In diabetic cardiomyopathy model, H19 inhibits autophagy by epigenetically silencing of DIRAS3. H19 knockdown could reduce EZH2 occupancy and H3K27me3 binding in the promoter of DIRAS3 (Zhuo et al., 2017). Perhaps the most dramatic finding of the large scale (genome wide) epigenetic effect of H19 was reported by Zhou et al. (Zhou et al., 2015). In this report, it was shown that H19 binds to and inhibits S-adenosylhomocysteine hydrolase (SAHH), the only mammalian enzyme capable of hydrolysing S-adenosylhomocysteine (SAH). This enzyme is a potent feedback inhibitor of S-adenosylmethionine (SAM)-dependent methyltransferases that methylate nucleic acids, proteins and lipids. SAHH modulation by H19 thus exerts global effects by causing methylation changes at numerous gene loci genome-wide. This represents the first case in which H19 acts in trans to alter the epigenetic landscape genome-wide.

The influence of H19 lncRNA extends beyond the cells transcribing it

With the discovery that RNA could be secreted outside the cells through exosomal vesicles, and cause phenotypic change in the cells receiving them, major changes about the local (cells transcribing it) phenotypic effect of these transcripts have been challenged with a very long believe that this function is only attributed to proteins. Cells communicated through RNA exosomes is relatively novel and could happen between normal cells, between cancer cells, and between normal and cancer cells. H19 lncRNA has functions far outside the cells transcribing it.Multiple reports indicate that exosomal H19 can induce drug resistant. For example exosomal H19 facilitated erlotinib and gefitinib resistance in non-small cell lung cancer (NSCLC) (Pan et al., 2020, Lei et al., 2020), H19 is delivered by exosomes to sensitive cells, leading to the dissemination of doxorubicin resistance in breast cancer (Wang et al.,2020). Carcinoma-associated fibroblasts promote the stemness and chemoresistance of colorectal carcinoma by transferring exosomal H19 (Ren et al., 2018). Additional reports have indicated that exosomal H19 induce other phenotypes related to tumorigenesis including stemness, angiogenesis, cell invasion and migration and proliferation. Interestingly many of those phenotypes are induced in recipient cells with similar H19 scenarios of action to those described in the previous sections. In summation, it is increasingly clear that the H19 lncRNA is a major player and a corner stone of many facets of the tumorigenic processes. It's an ideal target for therapeutic intervention. It uses diverse tools embedded in its primary RNA sequences to act as a sponger or a producer of microRNA (Figure 1).

Globally, H19 modify gene expression through epigenetic regulation genome wide. Despite this impressive progress made in the past decade, still we have many points need to be addressed for better understanding.We have highlighted the importance of H19 lncRNA during many stages of tumorigenesis. We are aware that the vast scenarios presented above are collected from different models and situations. So what dictates the mode of action? For instance, E-cadherin is suppressed by H19 by at least three modes of action as a sponger and a producer of microRNA, and also through epigenetic regulation.

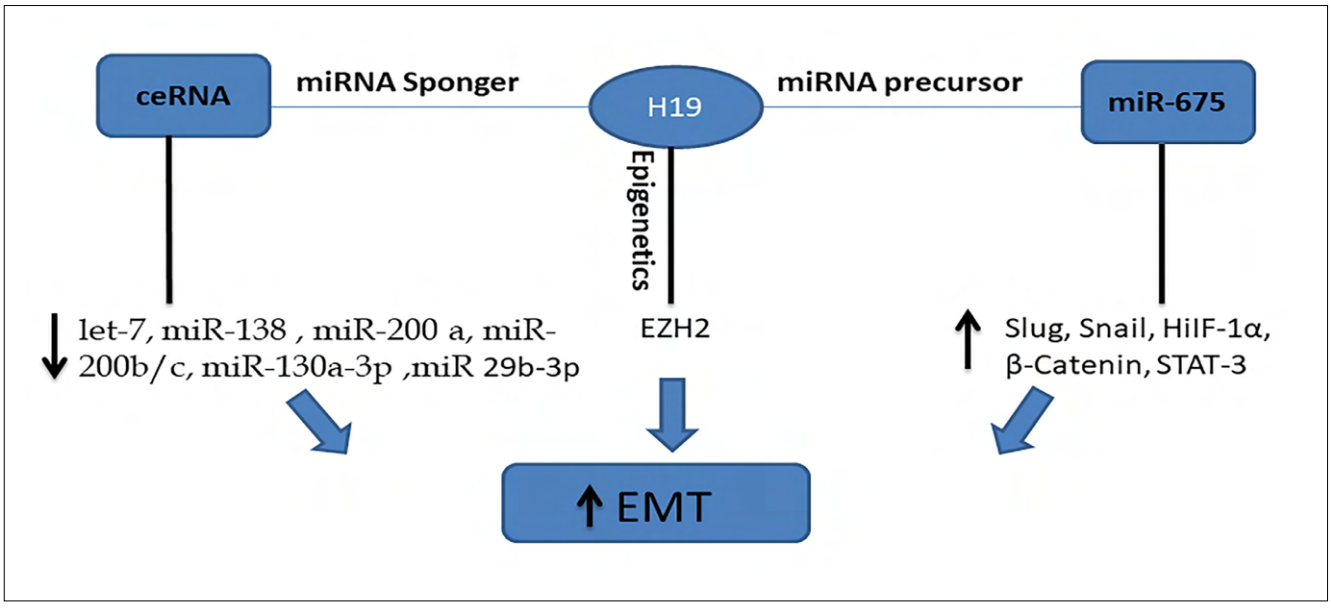

Figure 3: H19 lncRNA induces EMT via multiple mechanisms of action
By acting as competing endogenous RNA, miRNA precursor, and epigenetic regulator, H19 lncRNA induces EMT. Shown are some of the validated targets of H19 and its miRNA miR-675.

Are these scenarios happening concurrently in the same cancer cells to assure E-cadherin suppression or each scenario is happening in a tissue-specific manner? What triggers the release of H19 through the exosomes? Do H19 and miR-675 have different regulatory sequences, taking into consideration that they are both upregulated in many cancer types and it seems that miR-675 is not produced an the expense of H19. Why are still some reports arguing for a tumor suppressor function of H19 and its miR-675- Is H19 gene locus playing a dual role? If yes what triggers each role. Another question that needs to be addressed is whether H19 lncRNA is also acting as an antisense transcript given that its locus also transcribed in antisense direction.

Certainly, understanding the different mode of actions performed by H19 lncRNA will foster our thoughts towards delineating the possible roles and the mechanism of action of so many lncRNA with unknown function. It is not odd to speculate that the mechanistic mode of action of H19 is shared by other lncRNAs.

Competing interest

The author declares no competing interest to disclose.

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Prevalence of vitamin D and vitamin B12 deficiency in patients reporting to the West Bank governmental hospitals in the period between January 2015 and December 2018

RESEARCH

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A B S T R A C T

Vitamin D and vitamin B12 deficiencies are major public health problems; they may result from inappropriate low exposure to sunlight, autoimmune diseases or diminished intake. These two deficiencies have been extensively studied globally: causes, effects, treatment, as well as epidemiology. In Palestine the epidemiology of vitamin D and vitamin B12 deficiencies has not been addressed. This study was undertaken to determine the prevalence of vitamin D and vitamin B12 deficiencies in patients reporting to the West Bank (WB) governmental hospitals in the period between January 2015 and December 2018. It is a retrospective cross-sectional study for the data collected from medical records of patients tested for these deficiencies in 12 WB governmental hospitals for the three years period. Out of 30890 patients tested for vitamin D levels, 88% had insufficient vitamin D levels (< 30 ng/ml), whereas out of 43532 patients tested for vitamin B12, 19% had insufficient vitamin B12 levels (< 203 pg/ml). The percentage of patients with insufficient vitamin D levels is alarming. The percentage of patients with insufficient vitamin B12 levels falls within ranges reported by other studies in various countries. In conclusion, this study revealed an alarmingly high percentage (88%) of vitamin D deficiency below the reference sufficiency level among patients suspected to have such a deficiency. Around one fifth of the patients tested for vitamin B12 had insufficient levels. Because testing for vitamin D is costly, we suggest, that medical suspicion of vitamin D deficiency would be adequate to initiate treatment to alleviate the expense, especially in high-risk groups such as elderly women. Future studies have to address major risk factors contributing to these deficiencies that are specific to our community.

Keywords: Vitamin D, Vitamin B12, Deficiency, prevalence, West Bank, Palestine.

Introduction

Vitamin D and vitamin B12 play critical roles in the human body and in its development. Vitamin D was previously associated with calcium absorption and healthy bones only. Nowadays, with more recent studies showing that the vitamin D receptor is present on almost all cell membranes (Szabó 2011), we understand that it has more sophisticated functions. For example, potent inhibition of cellular growth and renin production, stimulation for insulin release, intrinsic factor production, and immune modulation (Haroon & Regan, 2010). Vitamin D deficiency leads to a variety of conditions starting with mild muscle pain and weakness to increased risk of osteoporosis or worse yet cancers and autoimmune diseases (Sahota, 2014). Vitamin D3 is obtained from the diet and from sunlight exposure. It is formed in the skin as a result of exposure to UBV radiation between 280

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and 315nm wavelengths on 7 dihydrocholestrol, a substance normally found in the skin. It is then converted to 25-hydroxyvitamin D (25(oH)D) in the liver; the storage form, which is the form used as an indicator for serum vitamin D status. Further metabolism occurs in the kidneys giving the biologically active form 1,25 hydroxyvitamin D (1,25-(oH)D) also known as calcitriol (Sahota 2014). Vitamin B12 functions in myelination of the nerves and long-term maintenance of the nervous system. It also has a major role in red blood cell formation. Vitamin B12 deficiency leads to megaloblastic anemia and combined subacute degeneration of the spinal cord, which manifests as general weakness, tingling or numbness in hands and/or feet and confusion (Oberley & Yang 2013). Vitamin B12 (cobalamin) cannot be synthesized in the human body and needs to be obtained from the diet such as meat, eggs and dairy products (Green et al., 2017). There are two assays used for measuring 25(oH)D in the serum. The golden test is by employing chromatography, the other is immune-based assay, which is the most commonly used assay in clinical practice (Sahota 2014). The cutoff point to determine deficiency or insufficiency of vitamin D remains a controversy; different studies use different reference levels.

The Institute of Medicine (IOM) report in 2011 indicated that adequate levels of vitamin D range between 50 -125 nmol/l (20 -50 ng/ml) (Ross et al., 2011). In this study, based on the cutoff points by the IOM for vitamin D, levels lower than 75 nmol/l (30 ng/mL) were considered as insufficient, lower than 50 nmol/l (20 ng/mL) as deficiency levels, and higher than 125 nmol/l (50 ng/mL) as harmful levels. However, clinical deficiency only shows in levels lower than 25 nmol/l (10 ng/mL). To diagnose vitamin B12 deficiency, total serum vitamin B12 concentration is measured. Different cutoff values are used to determine vitamin B12 status. However, according to the WHO, the cutoff value suggested for defining vitamin B12 deficiency is < 203 pg/ml (150 pmol/L) serum vitamin B12. In this study, the normal reference range for serum vitamin B12 will be considered to be between 200 - 900 pg/ml (Hannibal et al., 2017). In Jordan, one-third of the adult population (age >18 years) suffer from vitamin B12 deficiency (levels below 200 pg/ml) with no difference between males and females (El-Khateeb et al., 2014). Vitamin D and vitamin B12 deficiencies appear to be a pandemic phenomenon, but still not well characterized in Palestine, thus this study is an attempt to provide more insight into the status of these two vitamins in the Palestinian population.

This study aimed to fill the gap between public perception of the extent of this health problem and actual data. Lastly, it is an effort to direct the spotlight on the wealth of data, the Palestinian health care system has and to illustrate how this rich pool of data can be utilized for studies that would altogether give a clear picture of the health status in Palestine.

Materials and methods

This study is a retrospective cross-sectional study. The data was collected from twelve governmental hospitals' medical records covering the northern, central and southern governorates. The data was kindly provided by the information technology department in the Palestinian ministry of health. The collected data included all vitamin B12 and vitamin D test results during the period between Jan 2015 to Dec 2018, along with the demographic patients' data including age, gender, and city of residence. The collection was done separately for each vitamin and for each year.

Permission to collect this data was obtained from the health education department, ministry of health (letter number: 162/1881/2018 on 19/11/2018). The total number of data collected for vitamin D and vitamin B12 was 32994 and 48191 respectively. The data was filtered by removing items that did not include all the targeted information; leaving 30890 valid items for vitamin D, and 44916 for vitamin B12. Additional filtering for vitamin B12 data was done by removing the outliers (any test result above 950 pg/ml or below 50 pg/ml). This was necessary because high levels (>950 pg/ml) may indicate assay technical failure, liver disease or occult malignancy etc; while levels below (50 pg/ml) can be seen in pregnancy, folic acid deficiency

etc. (Oberley & Yang 2013). The final sample size for Vitamin B12 was accordingly 43532. The details on outliers are provided in Table 1.

Table 1: Vitamin B12 data distribution among hospitals

Hospital	Sample size before removing outliers	Number of outliers	Percentage of outliers	Sample size after removing outliers
W	1155	51	4%	1104
BJ	5612	179	3%	5433
D	4189	89	2%	4100
H	12181	507	4%	11674
J	3485	124	4%	3361
Jr	3852	100	3%	3752
R	10321	227	3%	10094
N	1544	45	3%	1499
S	384	17	4%	376
T	887	14	2%	873
TL	848	24	3%	824
Y	458	16	4%	442
Total	44916	1393	-	43532

All the data was analyzed using Statistix 2.0

Results

Vitamin D Results

The sample size collected from hospitals' records, examined for vitamin D status after filtering was 30890. Females represented 71% (22060) of the sample with a mean ± SD of (16 ng/ml ± 15) Vitamin D level, 88 % of which were below normal levels (30-49.9 ng/ml). Males represented 29% (8830), with a mean ± SD Vitamin D level of (18 ng/ml ± 15.5), 89 % of which were below the normal levels.

Eighty eight percent of all the samples tested had a vitamin D level below normal (30 ng/ml), with the following distribution: 15% with insufficiency (20-29.9 ng/ml), 31% with deficiency (10-19.9 ng/ml) and 42% with clinical deficiency (<9.9 ng/ml) as shown in Table 2.

Table 2: Distribution of Vitamin D status (ng/ml)

	Clinical deficiency (<9.9 ng/ml)	Deficiency (10-19.9ng/ml)	Insufficiency (20-29.9ng/ml)	Normal (30-49.9 ng/ml)	High (>50ng/ml)	Total
Number of patients tested	12867	9510	4818	2900	795	30890
Percentage	42%	31%	15 %	9%	3%	100%
Mean of vitamin D ng/ml	7	14	24	37	71	16
Std. Deviation	2	3	3	5	60	16

Table 3: Vitamin D status (ng/ml) trend within years (2015-2018)

Year	2015	2016	2017	2018
Number of patients tested	387	4705	11972	13862
Mean of vitamin D ng/ml	16	15	16	17
Std. Deviation	11	13	15	19

As shown in Table 3, the number of patients who were tested for vitamin D deficiency increased from 387 in 2015 to 13862 in 2018 which represents 35.8x fold, with an insignificant improvement in vitamin D deficiency from a mean ± SD of (16 ng/ml ± 11) in 2015 to (17 ng/ml ± 19) in 2018.

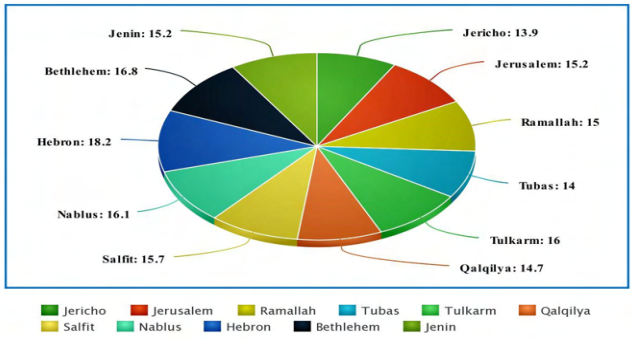

Figure 1: Vitamin D status (ng/ml) trend within WB cities

Further description of the vitamin D status (ng/ml) according to the city and the age of the patients tested are shown in Figures 1 and 2 respectively.

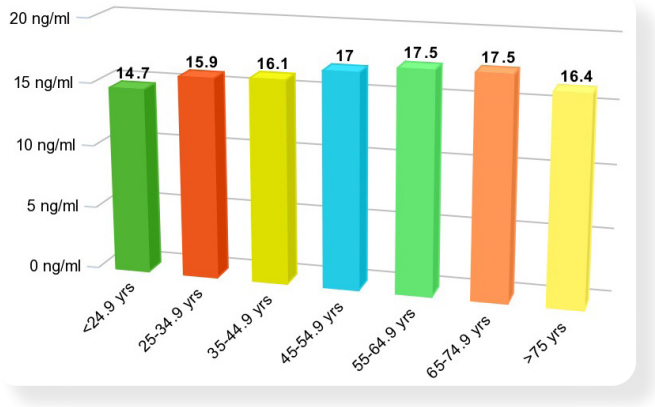

Figure 2: Vitamin D status (ng/ml) trend within age groups

Vitamin D status (ng/ml) mean in all age groups was less than (20 ng/ml) indicating deficiency, with no significant difference as referred to the patient's city origin.

Vitamin B12

The number of patients examined for vitamin B12 deficiency and included in this investigation was 43534. Females represented 66 % (28769) with a mean of vitamin B12 level pg/ml ± SD, (335 pg/ml ± 161), 18.6% of the females with deficiency levels, while males represented 34 % (14763) with a mean of vitamin B12 level pg/ml ± SD, (328 pg/ml ± 161) with 19.7% with a deficiency level. Out of the total (43534) tested for vitamin B12 levels, 158 (19%) had B12 deficiency (<199.9 pg/ml) as shown in Table 4.

Table 4: The distribution of vitamin B12 level (pg/ml)

	Deficient (<199.9 pg/ml)	Normal (200-899.9 pg/ml)	High (>900 pg/ml)	Total
Number of patients tested	8265	35050	219	43534
Mean of vitamin B12 pg/ml	158	370	924	434
Std. Deviation	30	145	15	63.3
Percentage	19%	80.5%	0.5%	100%

Table 5: Vitamin B12 levels (pg/ml) trend within years (2015-2018)

Year	2015	2016	2017	2018
Number of patients tested	4789	9751	12435	16559
Mean of vitamin B12 pg/ml	341	319	334	338
Std. Deviation	160	156	161	163

As shown in Table 5, the number of patients tested for vitamin B12 serum level increased from 4789 in 2015 to 16559 in 2018 representing a 3.5x fold. Further description of the vitamin B12 mean according to the city and the age of the patients tested are shown in Figures 3 and 4 respectively.

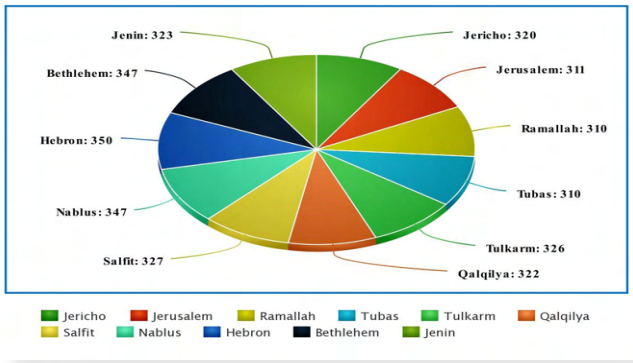

Figure 3: Vitamin B12 level (pg/ml) trend within WB cities

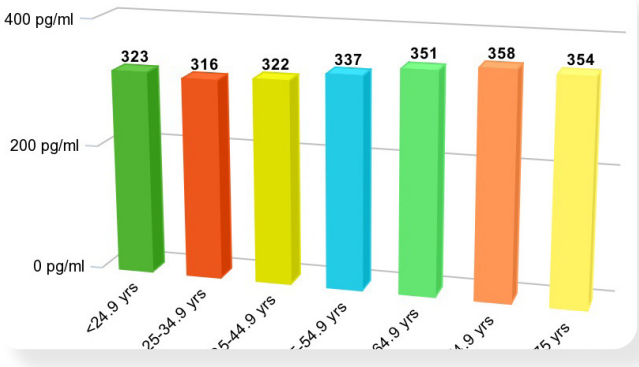

Figure 4: Vitamin B12 level (pg/ml) within age groups

Vitamin B12 mean levels (pg/ml) for all patients examined between January 2015 and December 2018 were within the normal range (Figure 3) and the same is true when the vitamin B12 levels in the patients from the different cities (Figure 4) were compared.

Discussion

This study shows the extent of vitamin D and Vitamin B12 deficiencies in patients reporting to the West Bank governmental hospitals in the state of Palestine during the period 2015 – 2018. The number of females examined for vitamin D deficiency was 2.4x fold greater than the number of males examined. The prevalence of deficiency between these two groups was similar, 88% below normal levels for the females and 89% below normal levels for the males. The mean level for vitamin D testing is (16 ng/ml ± 15), with 73% of the sample below the deficiency levels (10-19.9 ng/ml) and 88% below the sufficient levels (30-50 ng/ml). Previous studies on two subpopulations in Palestine similarly showed a high prevalence rate of vitamin D deficiency, in which 60.7% of the children were vitamin D deficient (Chaudhry, Hajat & Rizkallah, 2018). While the deficiency prevalence was 85.9% in postmenopausal women (Kharroubi, Saba, Smoom, Bader, & Darwish, 2017).

The prevalence of vitamin D deficiency in the neighboring countries is similarly high. In Syria, the prevalence of vitamin D deficiency is 61% (Sayed-Hassan, Abazid, & Alourfi, 2014), while in Jordan it is 60.3%(Nichols et al., 2012) and Saudi Arabia is 52.6% (Ardawi, Sibiany, Bakhsh, Qari, & Maimani, 2012). The overall vitamin D deficiency prevalence in the Middle East in a systematic review was found to be between 30-90% depending on the type of study, country, age etc (Bassil, Rahme, Hoteit, & Fuleihan, 2013).

In this current study, in the period between 2015 and 2018 there was a remarkable increase (35.8x) in the number of patients tested for vitamin D levels. However, there was a small increase in the mean of vitamin D (Table 3). This slight increase may be due to increased public awareness of vitamin D deficiency and the increased use of vitamin D supplements; as noted by different studies showing the increased use of supplements in different subgroups (Lips et al., 2019). However, other studies still point out that vitamin D supplements' use in the Middle East is not sufficient enough to fulfill the daily requirements of vitamin D (Hwalla et al., 2017).

Use of Vitamin D supplements without testing: where do we stand? In a recent study dedicated to understand the current situation of vitamin D deficiency and means of its prevention in Europe and the Middle East; it was evident that the daily intake of vitamin D in the Middle East is not sufficient enough to meet the required daily dose

of the vitamin. This same study recommended the use of vitamin D supplements in specific high risk populations including: children younger than the age of 1 year, pregnant women, persons living in institutions, persons older than the age of 70 and all non-Western immigrants (Lips et al., 2019). In the Netherlands, new vitamin D supplementation recommendations state that even adults with dark skin, or white skin adult but insufficient skin exposure should be taking vitamin D supplements (Weggemans, Kromhout, & Van Weel, 2013.

Two Middle Eastern countries; Saudi Arabia and United Arab Emirates have set recommendations for daily vitamin D supplementation taking into consideration the age and the reproductive status of the individual (Al-Daghri et al., 2017; Haq, Wimalawansa, Pludowski, & Al Anouti, 2018). However, the level of vitamin D supplement use in the Middle East is still low (Hwalla et al., 2017). In Palestine, the ministry of health recommends vitamin D supplementations for children from the age of 21 days until the age of 1 year. In spite of these recommendations, the prevalence of vitamin D deficiency in children as previously mentioned is 60.7% (Chaudhry et al., 2018). Recent studies have linked a genetic component to vitamin D concentration worldwide (Shea et al., 2009; Trummer et al., 2012). Some of the heritable polymorphisms linked to vitamin D deficiency level were evaluated in Saudi Arabia and further confirmed the link of two of them to vitamin D deficiency (Sadat-Ali, Al-Turki, Azam, & Al-Elq, 2016).

In the light of the aforementioned studies and recommendations, and also given the high cost for vitamin D testing in Palestine; the use of vitamin D supplementation without testing may be recommended for patients who are considered high-risk groups or patients with high clinical suspicion for vitamin D deficiency. Moreover, the risk of vitamin D excess intake is low (Flynn et al., 2009). Most of the cases of vitamin D toxicity are related to errors in fortification of food or formulation of supplements specifically when unlicensed supplements are used (Taylor & Davies, 2018). With the appropriate prescription and accurate explanation of supplement ingestion this problem can be avoided. Giving supplementation in the aforementioned subgroups of patients without testing might save the government and the individuals the expense of testing; which can be directed toward more comprehensive studies to evaluate the exact prevalence in the general population.

Vitamin B12 in comparison to vitamin D

The number of females tested for vitamin B deficiency was 1.9x fold greater than the number of males tested, however both groups showed similar deficiency prevalence; 18.6% for the females and 19.7% for the males. According to a recent review about vitamin B12 deficiency, asymptomatic vitamin B12 deficiency ranges between 2.5% to 26% (Green et al., 2017).

In this study, 19% exhibited vitamin B12 deficiency, which falls within the general population range. The number of patients tested for vitamin B12 was larger than those tested for vitamin D. This surprising difference in deficiency levels of vitamin B12 and vitamin D is not unique and has been observed before (Yazici et al., 2019). However, it further emphasizes the need for more studies in this area for better understanding and improved management of these health problems. Highly noticeable for vitamin B12 results is the fact that the unfiltered data had abnormally high levels (above 2000 pg/ml) for vitamin B12, which may be an indication of lab error(s) or typing error(s) (Oberley & Yang, 2013); this may be due to several reasons including; under qualified lab workers, heavy workload on the laboratory technicians, or simply faulty equipment. This can be supported by the fact that the percentage of outliers was higher in certain hospitals more

than the others (Table 1).

This study is a description of the current governmental hospital data; and does not take into account factors such as disease state, dietary habits, or the use of vitamin D or B12 supplements at the time of the test etc. Furthermore, a reconsideration for testing patients prior to giving supplements of vitamin D, especially for the subpopulations with the highest risk such as elderly women, in consideration with the current difficult financial status of the Palestinians in general.

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Strategy for DNA extraction and detection from insect pests in stored home grain samples

RESEARCH

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A B S T R A C T

Stored grains are subjected to infestations with more than 60 species of insects, that responsible for millions of dollars loss and cause several health problems including allergies and gastrointestinal disorders. Traditional detection techniques are laborious, expensive and not sensitive to detect insect contamination at the egg and larvae stages. Therefore, alternative methods are needed for rapid and sensitive detection. One obvious approach is to develop a molecular approach utilizing genetic information of the potential insect species that infest grains for amplification of specific target gene fragment utilizing polymerase chain reaction [PCR]. In the present study, a number of known infested grain samples were used in standardizing a method to isolate larvae and adult insects that were based on centrifugation washing method and a filtration washing method. The isolated insects were subjected to DNA extraction and PCR amplification of defined regions of cytochrome oxidase I (COI) gene followed by sequencing to identify the different pest species. For PCR amplification new primers were designed and for this purpose the obtained COI sequences from different insects were aligned to design two sets of primers (named: COI-PCR4 and COI-PCR5) specific for the indicated insect mitochondrial COI gene. The designed primers were tested for their specificity and sensitivity. The suitability of PCR primers and DNA extraction methods were evaluated on eleven samples of commercial grains utilizing each primer set with the two extraction methods.

Keywords: Insect pests, grain, DNA extraction

Introduction

Grains are considered as the world's primary stable food and its seasonal harvesting obligates storage for different time periods either for short-term or long-term periods (Proctor, 1994; Rajashekar et al., 2010). During storage, grains are exposed to damage by microorganisms, mice and insect pests, which destroy about 10-20% of agricultural products annually (Dragisic Maksimovic et al., 2015; Rajashekar et al., 2010) (Holst et al., 2000). Improper maintenance of storage temperature and humidity leads to insect development, which then leads to biological and chemical damage (Chattha et al., 2015), For these reasons it is important to examine grains periodically for early detection of insects in order to minimize grain loss.

Over 60 species of insects can infest stored grains (Jian, 2019). Beetles (order *Coleoptera*), moths (order *Lepidoptera*) and mites (class *Arachnida*) are the most common species that live in human food grains (Aspaly et al., 2007) (Collins, 2012; Garcia-Cela et al., 2019; Waongo et al., 2019). The principle pests stages that cause

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damage are adult and larval stages of beetles and the larval stage of moths (Aspaly et al., 2007; Collins, 2012) (Abd El-Aziz, 2011; Hafiz, 1983). The presence of insect pests in grains causes quantitative loss due to direct feeding of insects which reduces grain weight, nutritional value and qualitative loss by contaminating the grains with insect excreta, pupal cocoons, dead bodies and odors (Collins, 2012). Many health problems are associated with insect infestations such as allergies, diarrhea and others (Arbogast et al., 2000; Athanassiou et al., 2017). Some of these pests act as vectors for aflatoxin producing fungi (Hell et al., 2000). Other insects have been involved in the transmission of pathogenic bacteria such as *salmonella* and *Enterococcus* spp. (Crumrine et al., 1971). Animals also may be affected if their feed were contaminated with insects and mites (Channaiah et al., 2010; Hell et al., 2000).

Visual inspection, sampling and sieving are widely used for insect detection in grains (Aspaly et al., 2007). Other methods were developed to detect hidden infestations including staining of kernels to detect eggs, density separation (Shi et al., 2016), x-ray micro-tomography (Toews et al., 2006), acoustical sensors technique (Mankin et al., 2010), near infrared spectroscopy (Perez-Mendoza et al., 2005), Enzyme-Linked Immuno-sorbent Assay (ELISA) (Dunn et al., 2008) and uric acid analysis (Wehling et al., 1984). The accuracy of these methods depends on insect species, their developmental stage and grain type (Abels and Ludescher, 2003; Dasmahapatra, 2010).

Molecular techniques were widely used to detect viruses, bacteria, fungi and insect pests with significant rapidity, reliability and allowed for large-scale analysis of multiple samples (Nowaczyk et al., 2009). This approach allows the detection of primary pests inside grain kernels after oviposition and during the early larval stages based on DNA barcoding using short DNA sequences from known region of the genome as a reference sequence for species identification (Abels and Ludescher, 2003; Dasmahapatra, 2010). DNA barcoding emerged as a rapid method for insect detection and identification by comparing unknown sequences against DNA barcodes for known species via distance-based tree construction or alignment searching (e.g., BLAST) (Min and Hickey, 2007; Virgilio et al., 2012). The standard sequence used was mitochondrial *cytochrome c* oxidase subunits *COI* and *COII*, *cytochrome b* or ribosomal DNA (Dasmahapatra, 2010; Virgilio et al., 2012).

The main focus of this study is to develop a reliable and specific molecular test for the detection of insect pests in home stored grains, standardization of suitable treatment for grains before DNA extraction and optimization of a convenient and efficient DNA extraction method suitable for insects found in plant seed.

Materials and Methods

Samples

A total of 11 grain samples including: corn, groat, lentils, rice, wheat, corn flakes, chickpeas, cumin, sesame, barely and animal feed were collected from local wholesale grocery stores. Samples were chosen randomly from sacks that comprised of 500 grams of large grains (<0.5cm) and 250 grams of fine grains (>0.5cm). Positive control samples were consist of rice, flour and barely samples that were heavily contaminated with fully developed larvae and adult insect pests. Insects' larvae and adult stages collected from infested flour, rice, and barely were isolated for DNA extraction and species identification.

Sample preparation for DNA extraction

All samples were processed using two procedures. 1-Centrifugation washing method: where 10 grams of grains were transferred to 50 ml sterile plastic tubes containing 20 ml distilled water followed by mixing for 2 minutes, then 10 ml of the turbid water were transferred into empty 50 ml plastic tube and centrifuged for 10 min at 4000 rpm. The supernatant was discarded and the pellet was collected for DNA extraction. 2-Filtration washing method: 50 grams of grains were transferred to sterile 100-200 ml glass beakers (according to grain size) containing 70 ml sterile distilled water, mixed for 2 min, then 40 ml of the turbid water were drawn and filtrated through 47 mm diameter and 8μm pore

size nitrocellulose membrane filters (Whatman Inc, Piscatway, NJ) using a vacuum filtration system. The membrane filters were left at room temperature to dry, punched and 4 small disks (0.5cm diameter) were taken for DNA extraction.

DNA extraction

The pellet or the filter discs were incubated in 1.5 ml tubes with 200 µl lysis buffer (50 mM NaCl, 10 mM EDTA, 50 mM Tris-HCl pH 7.4, 1% triton hours. Equal volume of TE-saturated phenol (pH 8) was added to the aqueous solution, vortexed for few seconds and then centrifuged for 2 minutes at 14,000 rpm. The upper aqueous layer was transferred to new tube and DNA was precipitated by 0.2 M NaCl and 2.5 volumes of 100% cold ethanol. The mixture was incubated overnight at -20oC and centrifuged at 14,000 rpm for 10 minutes. The DNA pellet was left to dry then it was suspended in 100 µl of sterile double distilled water and stored at -20°C until further use.

Primers Design

At the beginning; three different PCR systems (COI-PCR1, COI-PCR2, and COI-PCR3 were designed that amplify *cytochrome oxidase I* (*COI*) gene of Diptera insects (Table 1), these PCR systems were used to amplify *COI* gene from extracted DNA of larvae and adult insects isolated from rice, flour and barely infested samples. The amplification products were subjected for DNA sequence analysis for pests species identification using BLAST generated comparison with their original *COI* gene DNA sequences. Based on new *COI* gene DNA sequence of the identified pests, new primers were designed from regions of highly conserved sequences of *COI* with the assumption to amplify *COI* gene from many other pest species (Table 1).

Polymerase Chain Reaction (PCR)

The amplification reaction was carried in 25 µl final volume using 2x concentrated green-*Taq* DNA polymerase (Thermo-Fisher, USA), 15 pmoles of each primer (reverse and direct), 2 µl of the original DNA extract or 5µl of the 1:10 diluted sample. The amplification protocol was run as follows: 5 min at 95 °C followed by 35 cycles of 30 seconds at 95 °C, 30 seconds at 50 °C, 1 min at 72 °C, and a final elongation step at 72 °C for 10 min. The amplified DNA fragments were resolved on 1.5% agarose gel in TAE buffer (40 mM Tris, 20 mM acetic acid, 1mM EDTA).

DNA Purification and DNA sequencing

PCR amplified DNA fragments were purified by PCR purification kit (Qiagene, Germany) according to the manufacturer instructions. The purified products were sequenced based on dye terminator method, using an automated DNA Sequencer machine (AB477).

Table1:DNA sequence information of the used primers

PCR system	Primer Direction	Primer sequence (5'-3')	Amplicon size(bp)	Tm (c°)
COI-PCR1	Forward	TCATAAAGATATTGGAACTTTATAC	750	53.1
	Reverse	GATGTCCAAAAAATCAAAATAAAT		50.7
COI-PCR2	Forward	GGAACTGGGTGAACAGTTTATCCCCC	350	66.4
	Reverse	ATGTTGATAAAGAATAGGATCTCCTCC		60.4
COI-PCR3	Forward	AATAATATAAGATTTTGACTTCTTCC	350	52.8
	Reverse	TATAGTAATAGCTCCAGCTAAAACTGG		52.8
COI-PCR4	Forward	ATTGGAGGATTCGGAAATTGA	456	52.0
	Reverse	CCTCCTGCTGGATCAAAAAA		55.5

DNA Quantification

DNA positive controls were quantified using a *NanoDrop* instrument (Thermo Fisher scientific Inc, Waltham, Massachusetts, USA). Most of the positive control DNA samples were in the range between 50 to 300 ng/μl while the DNA extracted from grain samples ranged between 400 ng to 2 μg/μl.

Results

Preliminary screening of insect grain pests

DNA was extracted from isolated adult insects infesting barely and flour samples (Figure 1), followed by PCR amplification using the three *Diptera* based PCR systems (COI-PCR1, COI-PCR2, and COI-PCR3).

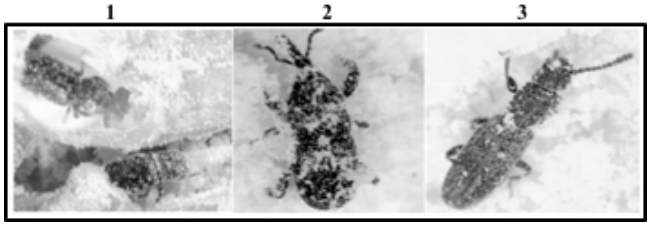

Figure 1: Different insect pest isolated from barely and flour infested samples which were identified according to their COI DNA sequence: (1) Barely: lesser grain borer (2) flour: granary weevil (3) flour saw-toothed grain beetle.

A successful PCR amplification was achieved by both COI-PCR1 and COI-PCR2 but not with the third PCR system (COI-PCR3) (Figure 2).

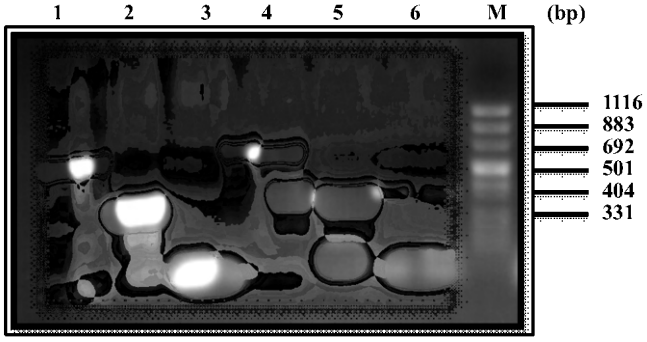

Figure 2: Agarose gel electrophoresis analysis of PCR amplified product of insect's DNA extracted from infested rice sample. 1 and 4: COI-PCR1, 2 and 5: COI-PCR2, 3 and 6: COI-PCR3. (M) Size marker.

DNA fragment about 700bp was amplified by COI-PCR1, and a fragment about 350bp was amplified by COI-PCR2. The amplified COI-PCR1 and COI-PCR2 DNA fragments from different unknown pests that were found in infested grains were sequenced. The sequence information was used to design more specific primers that are suitable to amplify *COI* gene from rice and grain pests (as described below).

Designing new specific primers

Based on the newly acquired *COI* DNA sequence information it was possible to identify pest type found in infested grain using BLAST sequence comparison. Table 2 shows a list of the major types of the identified pests and the similarity percentage to the obtained sequences. The DNA sequences of *COI* gene for the identified pests in table 2 were aligned using ClastalW2 software. The main purpose of this alignment is to identify a potential shared sequences for new primers that have the ability for a wider range of *COI* DNA gene amplification from many other pests species; and also a longer *COI* amplification that enables species identification after DNA sequence analysis. New direct and reverse primers that were used in PCR systems named: COI-PCR4 and COI-PCR5 were designed, these PCR systems amplify DNA segments of 456bp and 370bp respectively.

Table 2: Insect pest identified according to BLAST generated comparison

Pest scientific name	Pest common name	Accession number	Matching %
Plodia interpunctella	Indian meal moth	GU096541.1	99%
Oryzaephilus spp.	Grain beetle	KC407725.1	85%
Sitophilus spp.	Weevil	AY131101.1	76%
Rhyzopertha spp.	Grain beetle	KC407718.1	79%

Specificity analysis

The newly designed PCR systems (COI-PCR4 and COI-PCR5) were tested for their specificity. They did not amplify DNA extracted from insect free grains, plant leaves, and human DNA, even if 100 ng of DNA was used from each type of DNA (data not shown).

Sensitivity analysis

Serial dilutions ranged from 10ng to 1pg of DNA extracted from beetles were used to test the sensitivity of COI-PCR4 and COI-PCR5. It was clearly seen that COI-PCR4 is more sensitive than COI-PCR5; since it could amplify 1pg of the pests genomic DNA while the sensitivity of COI-PCR5 reached only to 10pg of DNA (Figure 3).

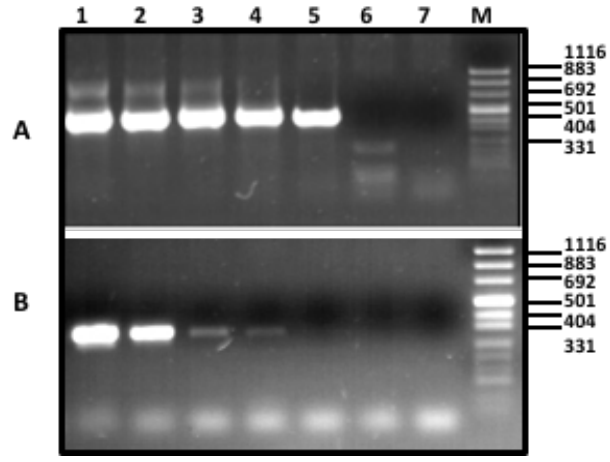

Figure 3: Sensitivity test of COI-PCR4 (A) and COI-PCR5 (B) targeting different concentration of beetle's pure genomic DNA isolated from barely. lane 1- 10ng, lane 2- 1ng, lane 3- 0.1ng, lane 4- 0.01ng, lane 5- 1pg, lane 6- 0.1pg, lane 7-Negative control, M: DNA size marker

Detection of pests in commercial collected grains

The two newly developed PCR systems (COI-PCR4 and COI-PCR5) were used to amplify pests' COI gene from 11 tested samples; after DNA extraction by filtration or centrifugation methods. PCR was performed with the extracted DNA and with DNA diluted 1:10. The results of the positive amplifications using both PCR systems with DNA extracted by the two indicated methods are summarized in table 3. COI-PCR4 system proved to be more efficient than COI-PCR5 in COI gene amplification from infected samples. Using this PCR system, it was possible to detect the presence of pests DNA in all 11 tested samples extracted by filtration washing method and after diluting 1:10. While using DNA extracted by the centrifugation method, it detects 10 out of 11 infected samples (Table 3, Figure 4).

The amplified COI fragment using COI-PCR4 system that target DNA samples from:

Table 3: Results of COI DNA amplification using COI-PCR4 and COI-PCR5 combined with centrifugation washing or filtration washing methods.

Extraction method	PCR system	Total positives original sample/ total	Total positives diluted (1:10)/ total
Centrifugation	COI-PCR4	6/11	10/11
	COI-PCR5	4/11	7/11
Filtration	COI-PCR4	9/11	11/11
	COI-PCR5	4/11	5/11

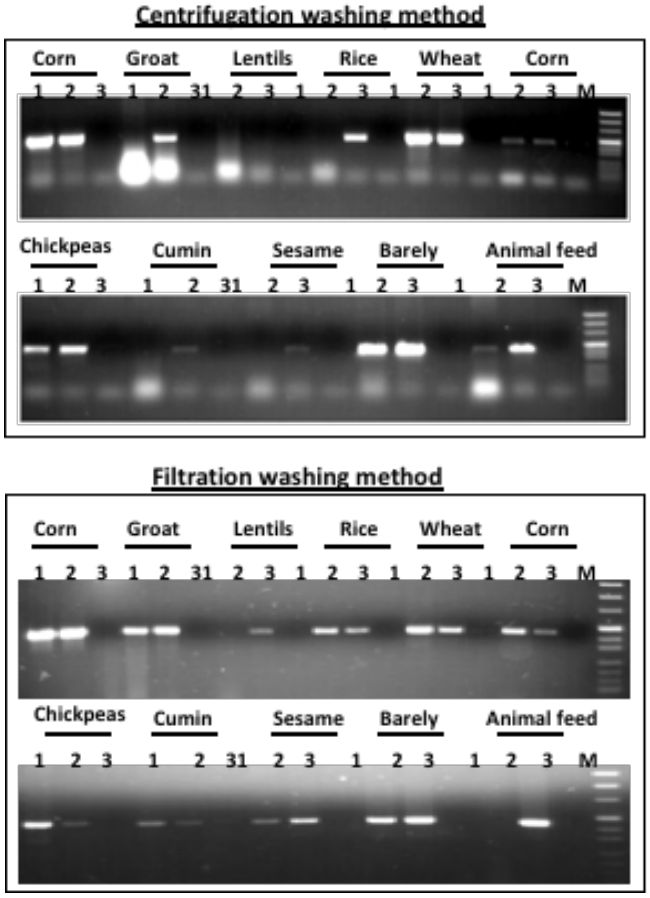

Figure 4: Agarose gel electrophoresis analysis of amplicons produced by COI-PCR4 primer system targeting DNA extracted from different types of grain samples by the centrifugation washing method (above) and filtration washing method (below). 11 samples were tested using three PCR reactions: 1- undiluted DNA, 2- diluted 1:10, 3- No DNA control. M: DNA size marker (bp).

corn, chickpeas, animal feed, rice and wheat extracted by centrifugation methodwere sequenced for species identification, the obtained

sequences were identified according to BLAST generated comparison as shown in table 4.

Table 4: insects identified according to BLAST generated comparison (amplification using COI-PCR4)

Pest scientific name	Source (sample)	Accession number	Matching %
Rhyzopertha spp.	Corn	KC407718.1	89%
Rhyzopertha spp.	Chickpeas	KC407718.1	74%
Samea spp. (*lepidoptera* spp.)	Animal feed	HM905018.1	84%
Rhyzopertha dominica	Rice	KC407718.1	99%
Rhyzopertha dominica	Wheat	KC407718.1	99%

Discussion

During shipping or storage periods grains are subjected to be infested by insect pests and mite, which cause economical losses by reducing grains quantity and quality. Hence, it is very important to adapt a sensitive method in order to detect insects at an early developmental stage or egg stage and implement control measures for the elimination of these insects and thereby reducing the loss of grains (Ngom et al., 2020). Insects can be found in many food components that if stored in cool dry conditions can slow further developmental stages that are hardly detected by the naked eye (Barrozo, 2019). In addition, many edible products are included in processed food as grind and milled materials infested with many types of insects that are hard to identify based on macro- or microscopic examinations (Abels and Ludescher, 2003; Hubert et al., 2018). Therefore, it is very important to develop a reliable sensitive diagnostic test that overcomes most classical insect detection methods even after processing. Consequently, detecting insects' genetic material provides a sensitive and specific examination tool for food and grain pests.

Previous studies using molecular approach dealt with one or two insect pests but lack the ability to detect several species of insects from different orders (Dasmahapatra, 2010). Our investigation focused on applying PCR amplification based on defined regions in the insects *cytochrome oxidase I* gene using general *COI* primers that are more specific for dipteral insects. Using this strategy, it was possible to detect and eventually identify different insects species in stored grains. DNA detection methods based on mitochondrial DNA is characterized by high sensitivity because of its many copies in the cell and approximately all mitochondrial genes sequences are known (Min and Hickey, 2007). The mitochondrial genes *COI* and *COII* subunits were used in standard molecular techniques to detect granary weevil in wheat flour (Ahrens et al., 2007). The DNA sequences of the amplified DNA fragments facilitated the design of more specific and sensitive amplification PCR systems for specific identification of insects in various grain samples.

Direct DNA extraction from grain samples by adding lyses buffer was not possible and several attempts were tried to overcome this problem, including the use of small sample size followed by short time incubation at high temperature for a quick extraction step. However, adapting this method for DNA extraction from plant or food materials for the purpose of insect examination was terminated since the small sample size can be misleading and many insect positive samples can be missed. These trials lead to the adaptation of two extraction procedures: 1- centrifugation washing and 2- filtration washing methods. Both protocols did not involve the addition of lyses buffer for long time in the presence of grain. This was achieved using a washing step with distilled water; and then collecting the wash water that contains particles; dust, eggs and fragments that were re-suspend in the lyses buffer. The filtration method relied on insect particles that will be returned on filter membrane with a pore size smaller than the smallest known pest egg (Wilson et al., 2003). Insect pest egg size ranges from 0.24-0.72 mm and the used membrane filter has a pore size of 8 μm. Another important complication was resolved after adopting these methods namely the dramatic reduction of plant DNA and proteins that may interfere in insects *COI* DNA amplification (Church et al., 2019).

All positive controls of insects DNA used in

our study were extracted from whole insects or their larvae that were directly removed from infested samples. Although it was possible to use previously identified *COI* DNA sequences for designing specific primers, we decided to have direct *COI* DNA sequences from our collected samples to identify specific types of insects isolated from local home or stores of grain samples (Min and Hickey, 2007). The developed COI-PCR4 and COI-PCR5 proved to be insect specific and did not amplify plant or human genomic DNA. These two systems also proved to be very sensitive, since it was possible to amplify 1pg of insect DNA template reflecting the sensitivity limits of these two systems. This sensitivity is equivalent to the detection of one egg found in 10 grams of grain sample (Ahrens et al., 2007).

Using classical PCR amplification, it is not possible to have quantitative results based on a single reaction. A quantitative procedure is needed to determine the insect accepted threshold level in grains according to the Federal Grain Inspection Service (FGIS) in the United States, which determines the number of insects allowed in grains (Fang et al., 2002). The combination of COI-PCR4 system and filtration system was sufficiently sensitive to detect even lower quantities than the allowed threshold level. Eventually, adapting these primers in a real-time quantitative PCR protocol would be even more sensitive for insect level detection.

The current developed molecular approach to detect insect pests in samples of stored grains were tested on eleven samples randomly collected from local stores. These samples were not purposely contaminated by insect pest for research purposes like previous studies (Hubert et al., 2018; Perez-Mendoza et al., 2005) but rather were processed as described using both centrifugation and filtration methods. The results based on using COI-PCR4 system in combination with centrifugation method shoed that ten of eleven collected grain samples were found to be positively infested. The results included positive amplification from corn, wheat, chickpeas, barely which gave very strong bands using the original and diluted samples, groat, rice and animal feed also gave strong bands for diluted samples only, lentils gave negative results whether using diluted or undiluted samples. The results indicated that using the filtration method for DNA extraction was more efficient and gave better results with the CO1-PCR4 system than the centrifugation method.

In conclusion, the data shown in this study represents a major step for the establishment of a rapid, sensitive and reliable molecular method for insect detection infested grains based on specific genetic marker information. Further work is needed to optimize the method for the specific identification of the various insects and develop a quantitative assay for the assessment the degree of contamination with the early developmental stages of insects for proper handling of contaminated grains. Furthermore, efforts will be directed to develop a multiplex test based on the present results for the detection of the vast majority of insects species known to infest grains. Eventually, this technique will hopefully open the way for the adoption of a national program to screen all imported and locally stored grains for periodic inspection to ensure the safety of grains for human consumption and prevents losses that can have a significant economical impact. Definitely, detection of insects contamination in grains at early stages will allow early interference to ensure the eradication of all contaminants utilizing effective and reliable methods that are used around the world for this purpose.

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Cluster analysis for food group consumption patterns in a national sample of Palestinian schoolchildren: Evidence from HBSC Survey 2013-2014

RESEARCH

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A B S T R A C T

Background: Promoting a healthy diet and lifestyle to reduce the national burden of nutrition-related problems among Palestinians requires an understanding of food consumption trends and patterns. Few studies have examined the food consumption patterns with the macro and micronutrient intakes and nutrition risk factors. The objective of this study was to study the food frequency and nutrient intake consumption patterns of Palestinian schoolchildren and their associations with the socioeconomic and risk factors.This is a national cross-sectional descriptive study conducted on Palestinian schoolchildren from the West Bank. The study examined the food consumption patterns of the macro and micronutrient intakes and nutrition risk factors among 1945 students aged 11-16 years. The data collected using the food frequency questionnaire and 24-hour recall that was administered by trained field workers. Food groups' classification, nutrient intakes, body mass index (BMI) Z-scores, and socioeconomic differences were examined across the food groups' patterns of consumption. We employed Z-score and K-Means cluster analysis to identify food consumption patterns and to examine factors associated with nutrient intakes. The food frequency results identified three food consumption clusters including the traditional, non-traditional, and mixed pattern. A total of 796 students (41%) were in traditional cluster, 458 (23.5%) in non-traditional cluster, and 691(35.5%) in mixed cluster. The nutrient intakes identified three clusters (High, Moderate, and Low consumption patterns) out of macronutrient, vitamins, and minerals categories. Most of the students located in the low consumption cluster for macronutrient, vitamins, and minerals clusters (66.9%, 67.7%, and 64 %) respectively. The traditional cluster was associated with healthy, non-obese, and physically active students and the non-traditional cluster was associated with unhealthy and obese students, but both shown significantly different across the identified clusters. Imbalance in dietary intakes among schoolchildren reflects a lack of dietary diversity. High sugar, fats and oils, and beverages consumption, low consumption of grains, fruits, beans and legumes, and meat are noticed in Palestinian schoolchildren. The findings indicated the importance of considering the food groups' intake variations among Palestinian schoolchildren. As the segments relate to children's health, nutrition diet programs should consider the high scores of non-traditional and mixed food consumption among schoolschildren.

Keywords: Cluster analysis; consumption patterns; nutrients; socioeconomic status; diet, food group patterns, schoolchildren.

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Introduction

Diet and lifestyle are major determinants of the health and development of chronic diseases. An unhealthy diet and sedentary lifestyle contribute to the increasing prevalence of obesity and non-communicable diseases like diabetes, hypertension, and cardiovascular

diseases, especially when adopted in childhood and early adolescence.(Farpour-Lambert et al. 2015; Mehio Sibai et al. 2011; Nishtar, Gluckman, and Armstrong 2016; Waters et al. 2014; James 2008). The consumption of an unhealthy diet is becoming more frequent due to the global rapid food intake transition (Salameh et al. 2014). This transition affecting the developing countries including Palestine. Change in socioeconomic status is associated with a transition in food consumption patterns across developing countries, where traditional healthy diets, including the Mediterranean diet, are being changed into more westernized dietary patterns. Moreover, in –society economic variation displays a difference in food consumption patterns. Individuals with high income consume more meat, poultry, fruits, and vegetables than those with lower income, who consume more saturated fats and carbohydrates(Heng and House 2018; Grieger, Scott, and Cobiac 2012; Nguyen et al. 2013; Alavian et al. 2008; Salameh et al. 2014). Schoolchildren and adolescents have been affected by the food consumption transition, a shift in diet consumption pattern is observed including high consumption rate of energy drinks, soft drinks, fast food, sweets and chocolates, and low consumption rate of fruits, and vegetables(Al et al. 2009; Aounallah-Skhiri et al. 2011; Williamson et al. 2020; Mehio Sibai et al. 2011). Several studies show that nutrition transition caused a high increase in childhood overweight and obesity rates. It has been found that unhealthy food is the leading source of calories, while healthy food such as fruits and vegetable intakes has decreased(Rahman et al. 2020; Aounallah-Skhiri et al. 2011; Williamson et al. 2020). Palestine is also subjected to nutritional transition (Abdeen et al. 2012; Aboul-Enein, Bernstein, and Neary 2017; Nubani-Husseini et al. 2016; Mikki et al. 2009). Similar to eastern Mediterranean countries, Palestine reported a high prevalence of overweight and obesity rates(Elessi and Albaraqouni 2019).

There are limited data on dietary intakes of Palestinian schoolchildren. Several studies have been conducted for assessing Palestinians eating habits, food consumption, and lifestyle including schoolchildren (Roblin 2007; Shah et al. 2019; Tariq, Shahid, and Tariq 2018; Zhou et al. 2016; Nubani-Husseini et al. 2016; Aboul-Enein, Bernstein, and Neary 2017). Investigation of transitions in food consumption and their relation to various sociodemographic variables is of paramount importance to analyze determinants of health and wellbeing among the population. Moreover, focusing on children and adolescents is important as this period is critical for adopting lifelong health behaviors. To date, limited information is available on food groups' consumption patterns among Palestinian schoolchildren. No published studies were found in which the food groups' consumption patterns were clustered and analyzed. This study acts as the first baseline study in which energy and nutrient intakes, lifestyle, and socioeconomic variables are compared according to dietary patterns for schoolchildren.

In Palestine, there is also limited information on the food groups' types and frequency that schoolchildren consume over a single week using FFQ data and over a single day using 24-hour recall data. Therefore, the present study aimed to (i) define the dietary clusters based on the frequency of consumption of food groups across a single week; (ii) define dietary clusters based on food group consumption across single day using 24-hour recall data (iii) compare energy and nutrient intakes clusters and lifestyle and physical activity variables between clusters.

Methods

Data were obtained from the national survey conducted in West Bank as a part of the Health Behavior in School aged Children (HBSC) survey in 2013-2014. The study aimed at improving the nutrition, physical and mental health of Palestinian children. Study subjects are Palestinian students in grades 5-9 (age 11-16) who were randomly selected from 100 schools in West Bank. The schools were randomly selected and stratified by school

type (55% Public and 45% UNRWA) and weighted according to population size. A random sample of 2000 students was selected from the baseline database weighted for gender and grades, out of 2000 students, 1945 students have accomplished the study criteria, the 55 students were excluded due to the incomplete data variables. Sampling procedures and methods have been described elsewhere in detail (Ziad Abdeen et al. 2018). The study received ethical approval from the Ministry of Education and Al-Quds University Institutional Review Board (IRB). The data were collected, entered, and cleaned by the Ministry of Education under the supervision of Al-Quds Nutrition and Health research institute (ANAHRI) at Al-Quds University.

Measure

The main outcomes of this analysis were the clusters of food consumption based on food frequency data and food intake and nutrient analysis. Dietary intake information was collected using face to face 24-hour food recall of one day intake and the validated food frequency questionnaire (FFQ) (Mikki et al. 2010). The 24-hour recall includes in-depth information about the food consumed during the last 24 hours. The participants were asked to recall the detailed descriptions of each food item consumed over the last 24 h including the quantity, time, and cooking descriptions. The food consumption quantities were identified using the recipes consumption weight book developed by ANAHRI. The participants' food intakes were entered and analyzed using the Nutribase V.9(Lee 1997), USDA, and the Palestinian Food Recipes databases. The Nutrient intake (Macro and Micronutrients) were computed using the Nutribase V.9 software. The dietary data collected from the 24-hour food recall generated a very large number of foods. The food items and recipes were classified according to the USDA food groups. Overall, the consumed foods were classified into standard food groups. The recipes were grouped by their main ingredients and depending on the ingredient that has the highest caloric value the food group was determined. As before this, the food group categorization methodology was determined based on a previous paper depending on the macronutrient composition of a food item, and 13 groups were set for this research purpose(Ahuja et al. 2012).

The classification produced 13 food groups: 1) vegetables; 2) fruits; 3) grains; 4) meat; 5) poultry and eggs; 6) fish and seafood; 7) beans and legumes; 8) dairy products; 9) sugar and sweets; 10) beverages; 11) fat and oils; 12) nuts and seeds; 13) Miscellaneous or others.

For the food frequency data, the food items were grouped into 8 categories based on similarity in nutrient profile (Frank et al. 1992). These categories were: 1) vegetables; 2) fruits; 3) milk and other dairy products; 4) sweets and chocolate; 5) soft drinks; 7) beverages (Juices with sugar); 8) energy drinks. Response categories were (1)never, (2) 1-2 times a week, (3) 3-4 times a week, and (4) 5-7 times a week (almost daily). As part of the HBSC survey, healthy and unhealthy nutritional practices were assessed using a frequency scale to obtain information on the nutritional status and variations among schoolchildren. Three items were considered indicators of "healthy nutritional practices", these items included the frequency of consumption of fruits, vegetables, and milk, respectively. Three other items were considered indicators on "unhealthy nutritional practices", these included frequencies of consumption of sweets, soft drinks, sugary juices, and energy drinks. Consumption frequency for all items was obtained over a week's duration.

The Demographic and other risk factors variables were collected using the HBSC unified tool(Al et al. 2009). The nutrient values were analyzed and compared to the USDA recommended Allowances (RDA) values for children aged 11-16 years old. The in-class administrative interview method was used for collecting the data. Students reported high response rates (97%) after excluding the cases with missing data.

Cluster Analysis

To identify dietary consumption patterns, we applied the K-Means clusters analysis to the data from the FFQ and the 24-hour recall. The consumption patterns were analyzed using the K-Means clustering algorithm for finding the segments of participants' food consumption. The K-means clustering defined the segments of data sets and assigned each observation into one K- distinct cluster, the algorithm identified the smallest variation within each data cluster, and the numbers of clusters were repeated until we found the best clustering match with the minimized square error between empirical means of the cluster and the points in the cluster. To identify the FFQ and energy and nutrient intakes clusters with similar patterns, the z-scores were calculated to standardize the nutrient data before clustering.

A non-hierarchical k-Means clustering procedure was used, with the random seed and 10 iterations to refine and optimize the classifications. The final cluster solution was selected based on interpretability and the percent of the study population in each cluster.

Statistical analysis

The statistical analysis was conducted using the IBM Statistical Package for Social Science V21. Furthermore, the dietary consumption patterns were analyzed for each cluster and descriptive comparison analysis was conducted between the consumption clusters for finding the patterns and the differences between clusters. K-Means cluster analysis was performed using SPSS V21. for Windows.

Chi-squared tests were used to assess differences between categorical data. Continuous data were assessed for normality and, if required, normalized with natural log transformation. One-way analysis of variance (ANOVA) was used to test for significant differences in mean nutrient and gram intakes between the clusters.

Results

Characteristics of respondents

The description characteristics of the study sample are presented in Table 1. A total sample of 1945 students was collected from the West Bank, Palestine. Among these participants around (47%) boys and (52.7%) girls. The mean age was 13.5 years, ranging from 11 to 16 years. The sample was selected from Public and UNRWA (Refugee) Schools. UNRWA represented (45%), and public schools represent (55%) of the study sample.

Table 1: The sample characteristics of schoolschildren 11-16 years old by gender.

Age in Years	Boys(n=920)	Girls(n=1025)	Total
		n (%)	
11-12	(38.6)355	(40.4)414	(39.5)769
13-14	(38.6)355	(37.7)386	(38.1)741
15- 16	(22.8)210	(22)225	(22.4)435
School Type			
Public	(48.7)448	(60.9)624	(55.1)1072
UNRWA	(51.3)472	(39.1)401	(44.9)873
Economic Status			
Low Income	(38)350	(31.7)325	(34.7)675
Moderate Income	(52.9)487	(57)584	(55.1)1071
High Income	(9)83	(11.3)116	(10.2)199
Living Place			
Refugee	(41.7)384	(27.4)281	(34.2)665
Non-Refugee	(58.3)536	(72.6)744	(65.8)1280
Father Education			
Secondary => School	(62.1)323	(69)459	(66)782
Secondary < School	(37.9)197	(31)206	(34)403
Mother Education			
Secondary => School	(62.3)294	(71.1)468	(67.4)762
Secondary < School	(37.7)178	(28.9)190	(32.6)368

Results in Table 2 show the lifestyle patterns of schoolchildren; the physical activity lifestyle was categorized into three levels (Low activity, Active and High activity) (17.1%, 56.5%, and 26.5%) respectively. Boys reported higher activity levels than girls (38%, 16%) respectively. About (72%) of boys spending more than 1 hour/day leisure time activity, compared to (68%) of girls spending >1H/day. Students reported higher poor healthy food than good healthy food consumption (53%, 47%) respectively. About (10%) of students smoking Nargila or cigarettes, boys were higher smokers than girls (14.7%, 5.5%) respectively. About (14%) of students were overweight and obese.

Table 2: Lifestyle characteristics of schoolchildren by gender.

Item	Boys(n=920)	Girls(n=1025)	Total
		n (%)	
Physical activity			
Low activity	89(9.7)	243(23.7)	332(17.1)
Active	480(52.2)	618(60.3)	1098(56.5)
High activity	351(38.2)	164(16)	515(26.5)
Leisure time activity			
<1hour/day	256(27.8)	368(35.9)	624(32.1)
1-2hours/day	396(43)	447(43.6)	843(43.3)
>3hour/day	268(29.1)	210(20.5)	478(24.6)
Healthy Food Consumption			
Poor	508(55.2)	522(50.9)	1030(53)
Good	412(44.8)	503(49.1)	915(47)
Smoking			
Yes	135(14.7)	56(5.5)	191(9.8)
No	785(85.3)	969(94.5)	1754(90.2)
BMI			
Underweight	62(6.7)	45(4.4)	107(5.5)
Normal	764(83)	804(78.4)	1568(80.6)
Overweight	66(7.2)	122(11.9)	188(9.7)
Obese	28(3)	54(5.3)	82(4.2)

The prevalence of overweight and obesity among boys and girls was (7.2%, 3%), and (12%, 5.3%) respectively. More than half of participants (55%) had moderate family income; (34.7%) had low family income and (10.2%) had high family income. In terms of living place, about (34.2%) living in refugee, and (65.8%) in non-refugee residence. Students reported the father and mother education with higher than secondary school were (66% and 67.4%) respectively, while father and mother education less than secondary school were (34% and 32.6%) respectively.

Energy and Macronutrient intake and food groups' consumption

The mean energy and macronutrient intake of Palestinian schoolchildren is reported in table 3. The overall mean energy consumption of boys was 2552 Kcal and 2064 Kcal for girls. The average consumption of protein for boys and girls was (89.2g, 69.6 g), respectively. The carbohydrate and fat average consumption for boys and girls were (360.3g, 292.9g) and (87.4g, 71.4g) respectively. The mean vitamins and minerals intake of Palestinian schoolchildren reported in Table 4. In the case of vitamins, boys is reported higher average consumption than girls in vitamin B1, B2, B3, B5, B6, B9, and B12 ((0.8,0.7); (1.2,0.9); (13.6,11.2); (3.9,3.1); (1.6,1.2); (273.4, 243.2); (301.1, 243.2); (3.3, 2.5)) (boys, girls) respectively.

The girls reported higher consumption of vitamin A and C than boys ((5.2, 5); (113.8, 99.6)) respectively. In the case of mineral consumption, boys reported higher average mineral consumptions including Calcium, Magnesium, Phosphorus, Potassium, Sodium, Iron, Manganese and Zinc. The boys in the age group (13-14 years) had a higher mineral consumption among boys. While girls in the age group 11-12 years had higher mineral consumption among girls.

Table 3: Mean (SE) of daily Intake of Energy and Macronutrients of Palestinian Schoolchildren.

Macronutrient	Boys(n=920)				Girls(n=1025)			
	11-12	13-14	15-16	All Ages	11-12	13-14	15-16	All Ages
Calories (Kcal)	2475(792.3)	2645.8(1109.9)	2525.4(843.3)	2552.4(940.2)	2133.9(712)	2025.3(725.6)	2002.5(720.6)	2064.2(720.7)
Protein(g)	87.6(35.8)	92(36.9)	87.5(38.1)	89.2(36.8)	72.2(30.1)	67.4(30.5)	68.6(31.6)	69.6(30.6)
% Protein	14(3.7)	13.8(3.5)	13.7(3.6)	13.9(3.6)	13.3(3.5)	12.9(3.2)	13.4(3.8)	13.2(3.5)
Carbs(g)	346.9(117.5)	376(230.3)	356.6(127.2)	360.3(172)	302.4(101.6)	286.8(102.3)	285.7(122.8)	292.9(107)
%Carbs	55.8(8.7)	55.6(8.2)	56.3(8.8)	55.9(8.5)	56.5(7.9)	56.5(7.9)	56.5(8.9)	56.5(8.1)
Fat(g)	85.2(37.3)	90.4(36.3)	85.9(38.2)	87.4(37.2)	73.8(33.1)	70.9(32.9)	68(27.5)	71.4(31.9)
%Fat	30.2(7.4)	30.5(7.3)	30(7.8)	30.3(7.4)	30.1(6.8)	30.6(6.8)	30.1(7.5)	30.3(7)
SatFat(g)	21.4(10.9)	23.4(11.7)	22.8(11.8)	22.5(11.4)	20.1(10.5)	19.2(11.3)	18.1(9.3)	19.3(10.6)
TransFat(g)	0.3(0.8)	0.3(0.8)	0.2(0.8)	0.3(0.8)	0.3(0.9)	0.3(0.9)	0.4(1.1)	0.3(1)
TransMonoFat(g)	0.1(0.3)	0.1(0.5)	0(0.3)	0.1(0.4)	0.1(0.3)	0(0.2)	0.1(0.3)	0.1(0.3)
MonoFat(g)	31.9(20)	33.6(18.9)	29.4(19)	32(19.4)	26.5(15.7)	24.8(15.4)	23.8(12.2)	25.3(14.9)
PolyFat(g)	17.9(10.1)	19(9.7)	19.6(10.9)	18.7(10.1)	15.6(8.8)	15.2(8.4)	15.6(8.3)	15.4(8.5)

Carbs: Carbohydrates, SatFat: Saturated Fatty Acids, TransFat: Trans-Unsaturated Fatty Acids, MonoFat: Monosaturated Fatty Acids, PolyFat: Polysaturated Fatty Acids.

Cluster Analysis

The food group consumption patterns were identified using the K-Means cluster analysis method. Several runs were conducted to identify the best pattern for nutrient intake and food frequency intake data. The nutrients intake was divided into three groups (macronutrient, vitamins, and minerals) clusters in addition to the FFQ clusters. The percentage distribution of clusters as described in table 5. Three clusters were found for each category. The FFQ clusters were identified as Traditional, non-Traditional, and Mixed. A total of 796 students (41%) were in traditional cluster, 458 (23.5%) in non-traditional cluster, and 691(35.5%) in mixed cluster. The nutrients intake clusters were identified as high, moderate, and low consumption (g/day or ml/day). Most of the students located in the low consumption cluster for macronutrient, vitamins, and minerals clusters (66.9%, 67.7%, and 64 %) respectively. The students in the moderate clusters were (26.4%,26.7%, and 26.2%). The students in the high consumption clusters were (6.6%, 5.6%, and 7.8%) respectively. The mean consumption of food groups in FFQ clusters is shown in table 6. The traditional cluster had the highest mean intakes of fruits, vegetables, milk, and milk products consumption. The Non-Traditional cluster had the highest mean intake of soft drinks, beverages, and energy drinks. The Mixed cluster had a high mean intake of soft drinks and sweets and chocolate groups. The univariant analysis shows the mean intakes of food groups varied significantly across the clusters. Table 7 shows the mean intakes of nutrients by FFQ clusters. Nutrient intakes varied significantly across the clusters. Students in the

Table 4: Mean (SE) daily Intake of Micronutrients (Vitamins and Minerals) of Palestinian schoolchildren

	Boys (n=920)				Girls (n=1025)			
Micro-nutrient	11-12	13-14	15-16	All Ages	11-12	13-14	15-16	All Ages
VitA(mcg)	330.7(67.6)	270.6(41.6)	164.4(12.6)	269.5(30.8)	189.7(32.3)	178.6(31.1	434(152.2)	239.2(37.8)
VitB1(mg)	0.8(0)	0.9(0)	0.8(0)	0.8(0)	0.8(0)	0.7(0)	0.7(0)	0.7(0)
VitB2(mg)	1.1(0)	1.2(0.1)	1.1(0)	1.2(0)	0.9(0)	0.9(0)	1(0)	0.9(0)
VitB3(mg)	13(0.5)	14.2(0.6)	13.4(0.7)	13.6(0.3)	11.1(0.4)	10.8(0.4)	11.8(0.6)	11.1(0.2)
VitB5(mg)	3.8(0.1)	4.1(0.2)	3.8(0.1)	3.9(0.1)	3(0.1)	3(0.1)	3.3(0.1)	3.1(0.1)
VitB6(mg)	1.3(0)	1.7(0.2)	1.6(0.1)	1.5(0.1)	1.1(0)	1.1(0)	1.2(0.1)	1.2(0)
VitB9(mcg)	286.5(10.5)	332.1(14.1)	273.4(11.3)	301.1(7.3)	246.2(8.4)	236.4(9)	243.2(12.6)	241.8(5.5)
VitB12(mcg)	3.8(0.6)	3.3(0.3)	2.5(0.2)	3.3(0.3)	2.4(0.3)	2.1(0.3)	3.4(0.6)	2.5(0.2)
VitC(mg)	92.1(5.3)	112.5(7.5)	90.8(5.7)	99.6(3.8)	117.1(6.9)	111.6(7)	111.5(9.5)	113.8(4.3)
VitD(IU)	57.8(3.1)	60.9(3.3)	75(5.5)	62.9(2.2)	57.8(3.1)	52.6(3.1)	52.3(4.2)	54.6(1.9)
Vit(EIU)	4.5(0.2)	4.9(0.2)	5.1(0.2)	4.8(0.1)	4(0.2)	4(0.2)	4.1(0.2)	4(0.1)
Ca (mg)	679.1(23.4)	692.2(25.1)	653.9(27.6)	678.4(14.7)	1013.3(386)	572.4(19.3)	555.1(25.9)	746.7(156.2)
Mg(mg)	279(8.3)	307.8(14.2)	257.6(9.7)	285.2(6.7)	355(128.4)	216.2(6.1)	212.5(7.2)	271.4(52)
P(mg)	1067.3(26.9)	1130.6(28.9)	1021.6(31.6)	1081.3(16.9)	843.5(18.7)	813.9(21.2)	815.7(26.3)	826.3(12.4)
K(mg)	2192.3(52.5)	2534.6(167)	2092.8(66.3)	2301.7(69.5)	1938.6(44.9)	1882.5(47.1)	1834.4(56.8)	1894.6(28.3)
Na(mg)	3816.7(81.8)	4180.7(85.3)	3964.5(108)	3990.9(52.2)	3837(524)	3305.4(75.4)	3154.9(87.6)	3487.1(214)
Cu(mg)	1.5(0)	1.6(0.1)	1.4(0)	1.5(0)	2.6(1.3)	1.2(0.1)	1.3(0.1)	1.8(0.5)
Fe(mg)	20.7(0.8)	21.5(0.8)	17.1(0.9)	20.2(0.5)	17.3(0.6)	15.8(0.7)	14.6(0.6)	16.1(0.4)
Mn(mg)	2.6(0.1)	2.8(0.1)	2.5(0.1)	2.6(0.1)	2.2(0.1)	2.2(0.1)	2.1(0.1)	2.2(0)
Zn(mg)	10.3(0.3)	11.2(0.3)	9.7(0.4)	10.5(0.2)	9.5(1.3)	7.6(0.2)	7.9(0.3)	8.4(0.5)

FFQ Clusters	Boys(n=920)	Girls(n=1025) n(%)	Total
Traditional	323(35.1)	473(46.1)	796(40.9)
Non-Traditional	267(29)	191(18.6)	458(23.5)
Mixed	330(35.9)	361(35.2)	691(35.5)
Macro-Nutrients Cluster			
High	57(6.2)	72(7)	129(6.6)
Moderate	269(29.2)	245(23.9)	514(26.4)
Low	594(64.6)	708(69.1)	1302(66.9)
Vitamins Cluster			
High	68(7.4)	41(4)	109(5.6)
Moderate	296(32.2)	223(21.8)	519(26.7)
Low	556(60.4)	761(74.2)	1317(67.7)
Minerals Cluster			
High	96(10.4)	56(5.5)	152(7.8)
Moderate	295(32.1)	254(24.8)	549(28.2)
Low	529(57.5)	715(69.8)	1244(64)

Table 5: K-Means clusters analysis for FFQ and nutrients intake food consumption.

Table 6: Mean (SE) Food Frequency Intake by the cluster.

Food Groups	Traditional	Non-Traditional	Mixed	F-Test (P<0.001)
Fruits	5.5(1.3)	5.1(1.6)	3.6(1.3)	0.0
Vegetables	5.5(1.3)	4.8(1.6)	3.7(1.4)	0.0
Milk and Milk Products	5.1(1.5)	4.1(2.0)	2.8(1.5)	0.0
Sweets and Chocolate	4.0(1.6)	4.9(1.8)	3.1(1.5)	0.0
Soft Drinks	3.2(1.5)	5.9(1.4)	3.5(1.7)	0.0
Beverages (Juice with Sugar)	4.1(1.6)	5.1(1.7)	2.9(1.4)	0.0
Energy Drinks	1.4(0.8)	4.2(2.2)	1.7(1.3)	0.0

non-traditional cluster show a higher mean intake consumption than other clusters in most of the Nutrients except Calcium, Sodium, Magnesium, and Copper where the mixed cluster reported higher consumptions of calcium, magnesium, sodium, and copper than other clusters.

The consumed grams of food groups

The mean (SE) grams of food groups consumed by the school students in each cluster are presented in table 8. Three clusters were identified for the grams consumed by students: The high cluster was 140 (7.2%) the moderate 556 (28.6%) and the low cluster 1249(64.2%). Girls in the three clusters consumed more beans and legumes, sugars and sweet, poultry and eggs, and fats and oils than boys. In the case of high consumption cluster, girls had a higher average consumption of poultry and eggs, beans and legumes, sugars and sweets, and fats and oils.

In the moderate cluster, girls had a higher consumption of fruits, meat, poultry and eggs, fish and seafood, beans and legumes and fats and oils than boys.

In the low consumption cluster, girls had higher vegetable consumption than boys. Significant differences were observed between food groups dietary patterns and gender. Girls' daily consumption of food groups was associated with significantly higher intake than boys in all food groups except fats and oils, nuts and seeds, and miscellaneous, where no significant differences were observed.

Energy and Nutrient intakes of consumed food groups

The nutrient intakes were divided into three groups: Macronutrients, vitamins, and minerals. The results of Z-score K-Means clusters identified three clusters for each group (High, Moderate and Low). Figure 1 shows the characteristics of macronutrient clusters, the percentage distribution of high, moderate and low clusters is (7.8%, 10.5%, and 81.1%), respectively. Children aggregated into cluster 1 had high Z-Score of energy, protein, and carbohydrate (2.6, 1.6 and 2.9), respectively. Children in cluster 2 scored moderate Z-score in energy, protein, and fat (0.7, 0.7 and 2.3), and had a negative score for carbs (Z-score =-0.4). Children in cluster 3 scored negatively on energy, protein, Fats and carbs z-scores were (-0.4, -0.3, -0.3, -0.2) respectively.

Characteristics of children described in the different clusters by the consumed food group are described in Figure 2. The macronutrient high cluster pattern (Figure 2a) included high fats and oils consumption (56%) of the sample and a significantly higher percentage of girls than boys were observed. In the moderate cluster pattern (Figure 2 b), children consumed a high percentage of grains (98%), there were no significant differences regarding gender. In the low cluster pattern (Figure 2c), children consumed more vegetables, sugar, and beverages (15.9%, 11.7%,

Table 7: Mean (SE) of schoolchildren nutrient intake by FFQ clusters.

Nutrient Intakes	Traditional	Non-Traditional	Mixed	F-Test-P-value
Calories(Kcal)	2354.4(33.3)	2386.5(39.7)	2166.2(29.4)	0
Protein(g)	80.8(1.2)	82.7(1.9)	74.1(1.2)	0
Carbs(g)	332.9(6.2)	335.2(5.7)	308.6(4.5)	0
Fiber(g)	23.9(0.7)	22.5(0.7)	21(0.5)	0
Fat(g)	81.4(1.2)	82.8(1.8)	73.7(1.3)	0
Retinol(mcg)	227.9(27.6)	333(76.7)	230.3(35)	0
VitB1(mg)	0.8(0)	0.8(0)	0.7(0)	0
VitB2(mg)	1.1(0)	1.1(0)	1(0)	0
VitB3(mg)	12.3(0.3)	13.6(0.5)	11.4(0.3)	0
VitB5(mg)	3.6(0.1)	3.8(0.1)	3.2(0.1)	0
VitB6(mg)	1.4(0.1)	1.5(0.1)	1.2(0)	0
VitB9(mcg)	277.3(7.6)	276.5(9.4)	256.9(7.1)	0
VitB12(mcg)	2.6(0.2)	3.4(0.3)	2.9(0.4)	0
VitC(mg)	117.6(4.9)	106.3(6)	95.6(4.4)	0
VitD(IU)	66.8(2.4)	56.7(2.7)	50.3(2.3)	0
Vit(EIU)	4.4(0.1)	4.6(0.2)	4.3(0.1)	0
Ca (mg)	682(15.6)	629.4(19.7)	808.1(23.2)	0.031
Mg(mg)	261.8(7.2)	257.2(7)	310.4(77)	0.005
P (mg)	974.6(17.1)	992.7(24)	884.6(16.2)	0
K(mg)	2222.6(78.7)	2082.6(46.1)	1934.1(35.7)	0
Na(mg)	3729.8(56)	3685.4(74)	3746.8(31.6)	0
Cu(mg)	1.4(0)	1.4(0)	2.1(0.8)	0.017
Fe(mg)	18.9(0.5)	18.1(0.7)	17(0.5)	0
Mn(mg)	2.5(0.1)	2.4(0.1)	2.3(0.1)	0
Zn(mg)	9.3(0.2)	9.7(0.3)	9.4(0.8)	0

and 12%) respectively. Significant differences regarding gender were observed. Figure 3 shows the characteristics of vitamin clusters, the percentage distribution of high, moderate and low clusters is (8.1%, 9.6%, and 82.3%), respectively. Children aggregated into cluster 1 had high Z-Score of Vit B6, B3, and B5 (2.8, 2.7 and 2.1) respectively. Children in cluster 2 scored moderate Z-score in B2, B3, and B5 (2.1, 1.3 and 1.2) and had low score for retinol (Z-score=0.1). Children in cluster 3 scored negatively on vitB6, retinol, B12, B1, B2, B3, and B5 (Z-score =-0.2,-0.07,

Table 8: The Mean (SE) grams intake cluster by food groups and gender.

Food Groups	Boys			Girls			Overall			P-Value
	High	Moderate	Low	High	Moderate	Low	High	Moderate	Low	
Vegetable	823.8	366.3	111.5	806.6	360.2	113.1	814.7	363.3	112.4	0.001
	(24.2)	(5.4)	(3.7)	(23.9)	(5.1)	(6.1)	(17)	(3.7)	(3.7)	
Fruits	804.7	344.5	144.4	779.4	345.4	129.3	794.3	345	135.5	0.001
	(29.6)	(7)	(13.8)	(29.3)	(6.3)	(4.1)	(21.1)	(4.7)	(6.1)	
Grains	771.8	391.5	211.7	708.9	354.2	153.1	755.6	372.2	173.4	0.001
	(15)	(4.6)	(29.5)	(19.9)	(4.1)	(3.4)	(12.4)	(3.1)	(10.6)	
Meat	643.9	290.7	75.1	605.4	316.8	72.1	634.3	297	73.5	0.001
	(18.4)	(7.9)	(2.9)	(0)	(21.5)	(2.7)	(16.2)	(8)	(2)	
Poultry and eggs	727.2	311.7	101.2	766.2	322.5	92.4	741.1	315.6	96.6	0.001
	(38.9)	(8.3)	(2.7)	(71.3)	(12.8)	(2.5)	(34.4)	(7)	(1.8)	
Fish and seafood	775	293	114.1	625.3	327	109.7	775	308	112	0.001
	(45)	(13.6)	(5.7)	(0)	(18.4)	(6)	(45)	(11.3)	(4.1)	
Beans and legumes	631.6	303	70.4	889.9	317.7	50.2	803.8	308	60.9	0.001
	(11.2)	(17.4)	(2.4)	(2.4)	(27.2)	(2.2)	(86.1)	(14.6)	(1.7)	
Dairy products	724.8	337.3	92.9	694	320.7	83.7	709.4	329.8	87.7	0.001
	(42.3)	(6.7)	(5.2)	(25.8)	(7.1)	(2.9)	(24.4)	(4.9)	(2.8)	
Sugars & sweets	425.2	278.2	37.8	1264.8	278.1	37.4	1264.8	278.1	37.6	0.001
	(15.7)	(21.5)	(1.4)	(0)	(19)	(1.3)	(27.1)	(14.6)	(1)	
Beverages	848	362	186.8	755.8	350.5	142.9	821.6	356	159.9	0.001
	(20.7)	(6.1)	(26.1)	(20.8)	(5.7)	(3.4)	(16.3)	(4.2)	(10.4)	
Fats & oils	328.4	256.5	47.5	468.1	390.7	35.9	382.2	290	41.6	0.07
	(18.2)	(17.4)	(1.1)	(18.7)	(63.9)	(0.8)	(11.3)	(28.1)	(0.7)	
Nuts & seeds	29.1	18.2	11.6	24.1	15.3	10.4	26.1	23.4	11	0.085
	(19.4)	(0.6)	(0.9)	(0.9)	(0.8)	(0.7)	(0.6)	(0.5)	(0.6)	
Miscellaneous	449.6	317.4	178.4	444.3	348.3	212.9	435.6	352.2	189.7	0.064
	(22.1)	(12)	(9.2)	(14.6)	(11.8)	(6.5)	(14.6)	(16.3)	(18.7)	

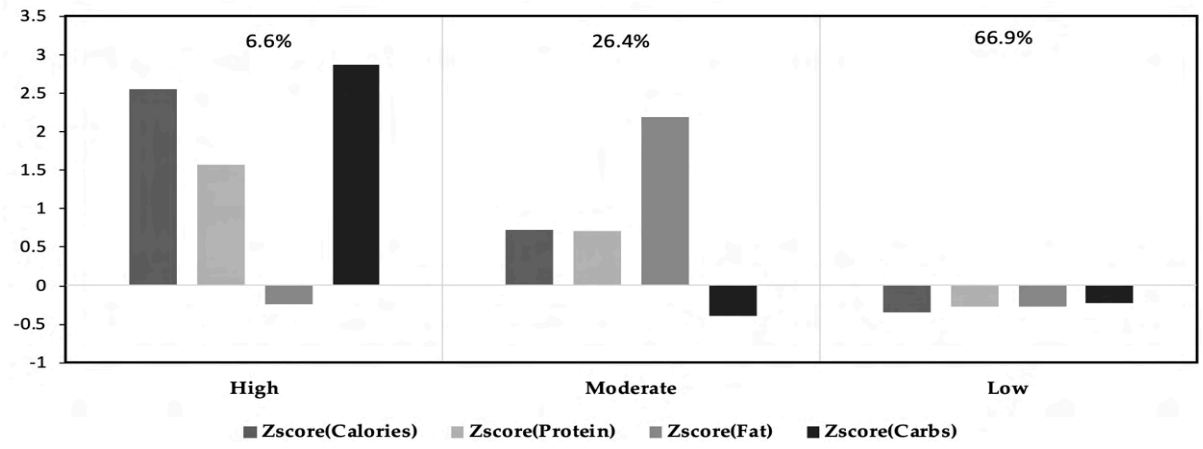

Figure 1: Average Z-score of macronutrient category K-Means clusters.

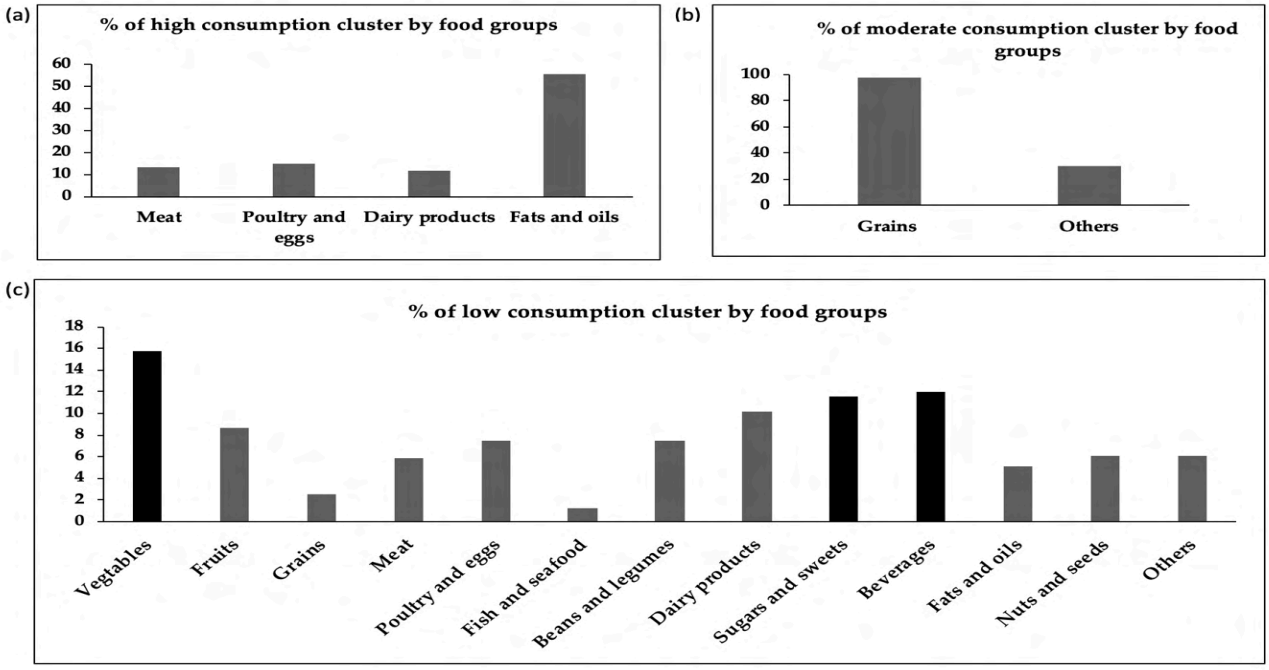

Figure 2: Percentage distribution of food groups consumption by Macronutrient category K-Means clusters.

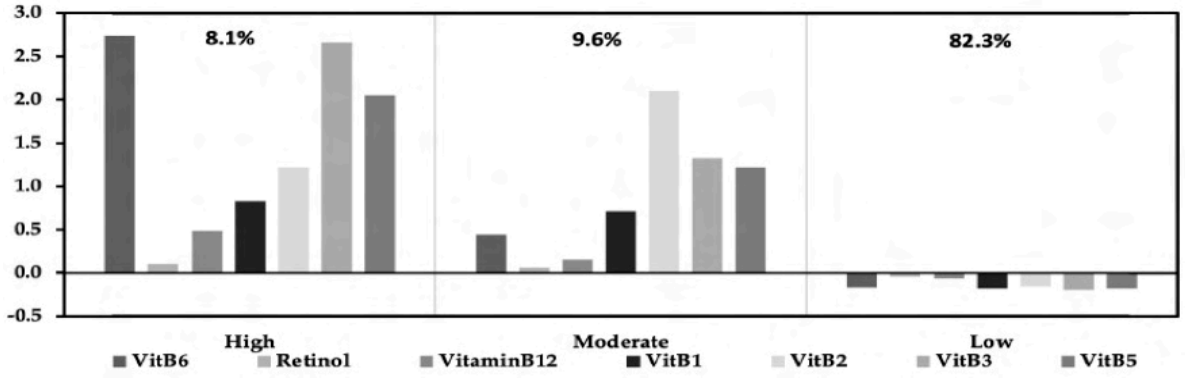

Figure 3: Average Z-Score of vitamin category K-Means clusters.

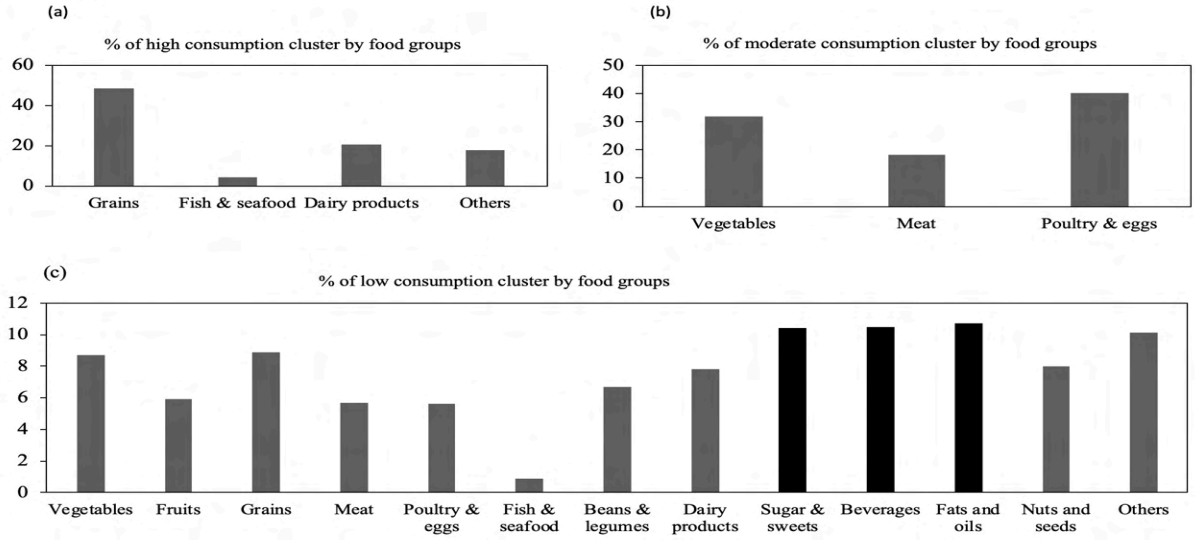

Figure4: Percentage distribution of food groups consumption by Vitamins category K-Means clusters.

-0.1, -0.2, -0.2,-0.3,and -0.2) respectively. Characteristics of children described in the different clusters by the consumed food group are described in figure 4. The vitamin high cluster consumed pattern (Figure 4a) included a high percentage of consumption of grains and dairy products (48% and 20%) of the sample and a significantly higher percentage of girls than boys did. In the moderate cluster consumed pattern (Figure 4b), children consumed high vegetables, meat and poultry and eggs (31%, 18%, and 40%) of the sample and a significantly higher percentage of girls than boys did. In the low cluster consumed pattern (Figure 4c), children consumed more sugar, beverages, fats and oils (10.1%, 10.2%, and 11%) respectively of the sample and a significantly higher percentage of girls than boys did. Figure 5 shows the characteristics of minerals clusters,

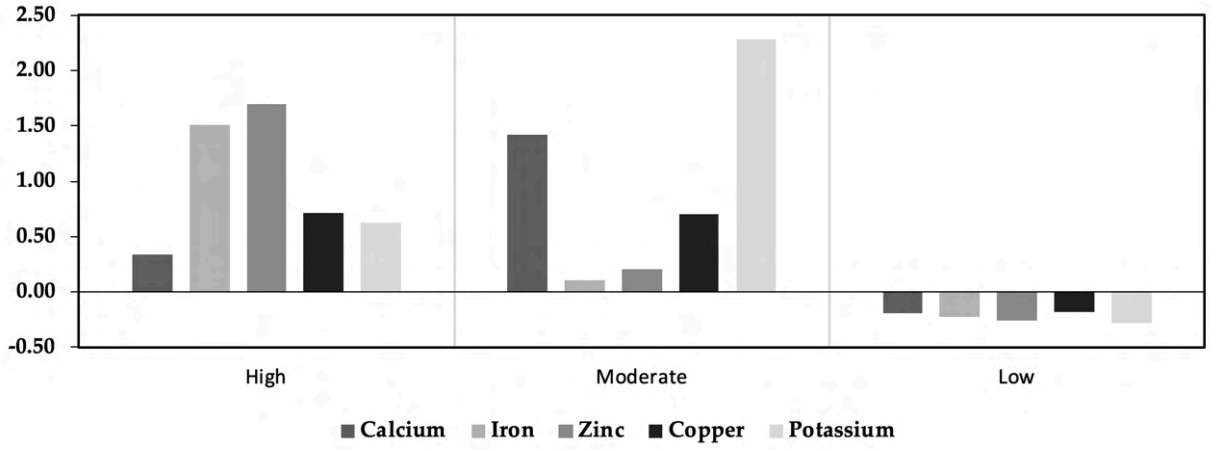

Figure 5: Average Z-Score of Minerals category K-Means Clusters.

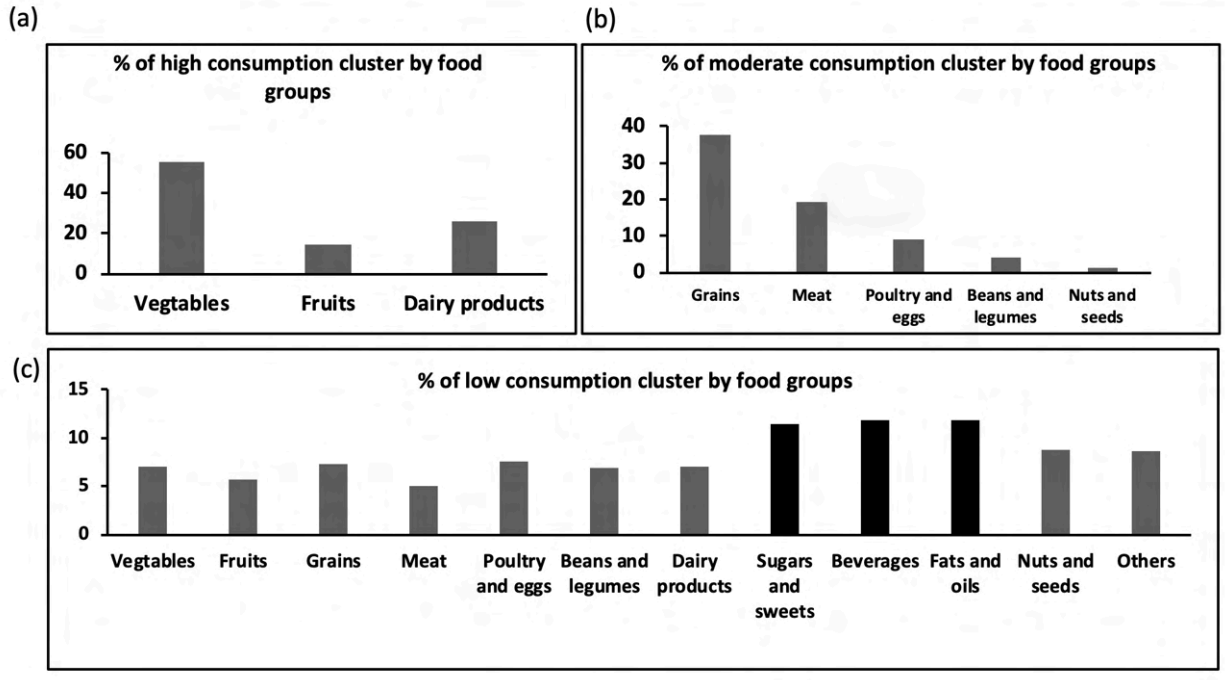

Figure 6: Percentage distribution of food groups consumption by minerals category K-Means clusters.

the percentage distribution of high, moderate, and low clusters is (3.8%, 18.9%, and 77.3%), respectively. Children aggregated into cluster 1 had high Z-Score of calcium, iron, zinc, copper, and potassium (0.3, 1.5, 1.7, 0.7, and 0.6 respectively. Children in cluster 2 scored moderate Z-score in calcium, zinc,copper, and potassium (1.4, 0.21, 0.7, and 2.3). Children in cluster 3 scored negatively on calcium, iron, zinc, copper and potassium (0.2, -0.22, -0.25, -0.18, and -0.28) respectively. Characteristics of children described in the different minerals clusters by the consumed food group are described in figure 6. The minerals high cluster consumed pattern (Figure 6a) included high vegetables, fruits, and dairy products (57%, 15%, and 27%) of the sample and a significantly higher percentage of girls than boys. In the moderate cluster consumed pattern (Figure 6b), children consumed a high percentage of grains and meats (48% and 19%), of the sample and a significantly higher percentage of girls than boys. In the low cluster consumed pattern (Figure 6c), children consumed more sugar, beverages, and fats and oils (11.5%, 11.9%, and 11.9%) respectively of the sample and a significantly higher percentage of girls than boys.

Discussion

The present study involves a national sample of schoolchildren aged 11-16 years-old from West Bank to assess the frequency of food consumption, the energy and nutrient intakes, and the patterns of food groups' consumptions. The study used K-Means cluster analysis to identify the consumption patterns, nutrient intake, and lifestyle differences. This study identified the consumption clusters and adds to the literature information about Palestinian children's food groups' consumption and nutrient intakes patterns, as well as how the clusters linked with lifestyle, physical activities, and demographic variables. Among all students, about 14% were overweight or obese, and more than half of the students corresponded to a moderate level of activity. Boys reported higher activity levels than girls. Furthermore, boys spent more time in leisure time activities than girls. A high portion of students consumed poor healthy foods including take-away, sweet, beverages, and energy drinks. Boys consumed higher unhealthy food than girls.

The results are consistent with findings by other authors reporting food frequency patterns among schoolchildren (Pérez-Rodrigo et al. 2015; Gharib and Rasheed 2011). The food frequency results indicated that students consumed high portions of vegetables, sweets, and soft drinks. Boys consumed higher energy and soft drinks than girls, while girls consumed higher sweets and chocolates than boys. Girls consumed more vegetables and fruits, while boys consumed more milk and milk products. The results of 24-hour recall reported the energy and nutrient intakes among school children. The study has its drawbacks of a one-time 24-hour recall that may not enough to represent the individual usual diet. However, it does represent the average daily consumption of students' groups because of the data analysis unaffected by person variation. The findings related to food frequency clusters are consistent with food consumption patterns in other studies (Desbouys et al. 2019; Williamson et al. 2020).

The mean energy intakes of Palestinian children were higher than RDA standards as well as values reported by USDA (Institute of Medicine 2009). The mean energy intake of children compared by other same-age students is a serious concern (Gharib and Rasheed 2011). High average of energy consumption contributed to high prevalence of overweight and obesity (Faught et al. 2017; Gharib and Rasheed 2011;

James 2008; Farpour-Lambert et al. 2015; Elessi and Albaraqouni 2019; Al et al. 2009). The average protein, carbohydrate, and fat intakes were above the RDA values about USDA standards. The macronutrients consumptions increased with age due to increasing intakes of soft drinks, sweets, chocolates, and energy drinks.

It is a serious concern that around 60% of students consumed at least one soft, or energy drink per day. The increase in the consumption of sugar has been associated with overweight and obesity(Grieger, Scott, and Cobiac 2012). Besides weight problems, sugar consumption is likely to decrease children's HDL cholesterol, increase LDL cholesterol, blood glucose, and insulin concentration factors which are related to Coronary Heart Disease mortality(Gerbens-Leenes, Nonhebel, and Krol 2010; Gharib and Rasheed 2011; Grieger, Scott, and Cobiac 2012). Furthermore, the increase in sugar consumption will cause the nutritional inadequacy of vitamins and minerals(Gharib and Rasheed 2011).

The results obtained in the present study matched previous studies in children and adolescents that used k-Means analysis to identify food consumption patterns, dietary intakes and lifestyle variables(Landsberg et al. 2010; Magee, Caputi, and Iverson 2013; Sanchez et al. 2007; Lioret et al. 2008; Sabbe et al. 2008; Gharib and Rasheed 2011; Grieger, Scott, and Cobiac 2012; Heng and House 2018). Cluster analysis groups students' intakes into mutually exclusive groups based on the similarity in food groups consumed, allowing clusters comparisons. The food frequency k-Means clusters produced three clusters classifying the food consumption patterns. The FFQ clusters in this study are consistent with findings by other studies reporting traditional, non-traditional and mixed consumption patterns that combine healthy and unhealthy food groups and assessed the relationship with physical activities and lifestyle.

Several studies have identified a healthier or traditional food consumption in children, with higher scores of vegetables, fruits, and dairy products(Rathnayaka, Selvanathan, and Selvanathan 2019; Kunin-Batson et al. 2015; Heng and House 2018; Landsberg et al. 2010). Other studies described non-traditional or mixed food consumption as unhealthy consumption patterns with high scores of soft drinks, sweets, and chocolates, and energy drinks(Gharib and Rasheed 2011; Magee, Caputi, and Iverson 2013; Grieger, Scott, and Cobiac 2012; Sanchez et al. 2007). Numerous studies assessed the combination clustering with lifestyle, physical activities, and a healthy diet(Aguilà et al. 2017; Magee, Caputi, and Iverson 2013; Sanchez et al. 2007; Lioret et al. 2008; Landsberg et al. 2010). Leech et al. (Rebecca M. Leech, McNaughton, and Timperio 2014)conducted a systematic review on the clustering of diet, physical activity, and sedentary behavior among children and adolescents aged 9–21 years, his study found that most of children and adolescents had mixed consumption pattern of healthy and unhealthy food. Another study also identified that a higher portion of girls aged 10-12 years old falls in low physical activity (Sanchez et al. 2007).

In our study, Z-scores K-Means clustering was used to identify the energy and nutrient intakes clusters among Palestinian schoolchildren. Three nutrient intakes categories were found, the macronutrient, vitamins, and minerals. Several studies used cluster analysis to identify the relationship between dietary intakes pattern and lifestyle (Jongenelis et al. 2020; Kulik et al. 2019; Pala, Reisch, and Lissner 2019; Shah et al. 2019; Nubani-Husseini et al. 2016).

The study extended other similar studies and identified three clusters (high, moderate and low) consumption patterns (Niermann, Spengler, and Gubbels 2018; Wirfält and Jeffery 1997; Grieger, Scott, and Cobiac 2012; Landsberg et al. 2010; Rebecca

M. Leech, McNaughton, and Timperio 2014; Sabbe et al. 2008). The macronutrient group identified approximately 81% of students with the negative and low z-scores cluster. Students in this cluster consumed more vegetables, sugar, and beverages. The vitamin group identified approximately 82% of students with low and negative scores, students in this cluster consumed more sugar, beverages, fats, and oils and vegetables. The minerals cluster identified approximately 77% of students with low and negative scores, the students consumed more sugar, beverages, and fats and oils.

It is expected that students in these ages consumed higher intake of sugar and soft-drinks and lower healthy nutrient intakes. Interestingly, there was a significant difference in nutrient intakes clusters with gender. Girls have a significant difference in consuming more vegetables in macronutrient groups and more sugar and chocolates in vitamins and minerals groups. The results in this study extended the results of other previous studies using cluster analysis that focused on dietary intakes, lifestyles, and sociodemographic status (R. M. Leech, McNaughton, and Timperio 2014; Gubbels, van Assema, and Kremers 2013; Hosseini et al. 2019; Sanchez et al. 2007; Rebecca M. Leech, McNaughton, and Timperio 2014; Sabbe et al. 2008). These studies have identified that schoolchildren consumed high energy food intakes and more unhealthy food groups, similar to these studies the Palestinian children highly exposed to takeaway food and soft-drinks, chocolates and energy drinks. The widespread of take-away food and sugar and energy drinks increased the students' accessibility to unhealthy food.

Difference between boys and girls in average grams consumption by food group dietary pattern indicated a significant difference by gender distribution, the boys consumed higher grams in vegetables, fruits, grain, meat, dairy products, and beverages, while girls had higher consumption in beans and legumes, fats and oils, and sugars and sweets groups. The food group consumption by gender was reported by other authors(Vasileska and Rechkoska 2012; Desbouys et al. 2019; Gharib and Rasheed 2011; Grieger, Scott, and Cobiac 2012). Gender difference in daily consumption was found, boys consumed higher vegetables, grain, beans and legumes, meat and poultry and eggs. Contradictory results have been reported regarding sugars and sweets, and fats and oils (Gharib and Rasheed 2011), boys consumed higher grams than girls, however, gender significant difference was found(Grieger, Scott, and Cobiac 2012).

The strengths of this study were found in the design of 24-hour food recall data. Food consumption data were analyzed using the Palestinian food recipes database and the Palestinian food composition table developed by ANAHRI. The food consumption classification according to the international food groups categories using energy and grams distribution was the first study among Palestinian schoolchildren. The cluster analysis using food frequency and nutrient intakes allowed us to identify the actual dietary patterns without any predefined criteria and their difference with physical activities, lifestyle and gender provided a comprehensive perspective.

There are some limitations to the present study, including the cross-sectional approach. Therefore, it provides evidence for the association but not causal relationships. Measures of food frequency consumption and physical activity relied on self-reports and were possibly biased, although a careful multistep quality control procedure was implemented under the supervision of the Ministry of the Education team to minimize bias. However, misreporting can influence the potential association with study variables. Cluster analysis is an observed method

of defining similar groups of individuals and is particularly appropriate for identifying groups that could benefit from interventions. A series of particular actions are required when carrying out a cluster analysis, including the selection of food groups, the variables used to determine clusters, especially the nutrient intakes clusters (e.g. grams of consumption, frequency of consumption, vitamins, and minerals), the number of clusters, and the clusters. These actions may not be appropriate in different populations. Thus, the repeatability and generalization of cluster analysis in different populations are limited. However, we performed the same cluster analysis methodology in the study population using the 24-hour face to face interview data, in which three similar clusters found. Using a single day's food intake may also misclassify individuals into a different cluster than what may occur after a longer-term intake. Finally, K-Means analysis is a procedure commonly used to identify dietary patterns and analyze the clustering of lifestyle. However, long term intake may provide more adequate clusters and better to identify patterns and clusters.

Conclusion

Three food consumption patterns were identified, traditional, none-traditional, and mixed, the traditional is close to the Mediterranean diet. Energy and nutrient intakes cluster analysis classified students into three groups with three clusters each. The macronutrient group, vitamins, and minerals. The clusters were identified as high, moderate, and low consumption scores. The lifestyle and physical activity identified two groups of students, unhealthier lifestyle patterns with low physical activity and high consumption of unhealthy foods; and healthier lifestyle patterns with high physical activity and high consumption of healthy foods. The significant differences were found in children into different clusters. Future research using larger samples is needed to further examine how food groups consumption and lifestyle patterns of nutrient intakes track over time and influence on children's health. These cluster analyses are helpful to identify Palestinian children's consumption patterns and act as a baseline for potential intervention strategies.

Declarations

Ethics approval and consent to participate The study received ethics approval from the Health Research Ethics Boards at the Ministry of Education and Al-Quds University. Written informed consent was obtained from all parents and written assent was obtained from children.

Availability of data and materials

The datasets generated and/or analyzed during the current study are not publicly available due to the current use by other Ph.D. students but are available from the corresponding author on reasonable request

Author Contributions

Conceptualization R.Q., Z.A, and H.S; methodology, validation, formal analysis, and writing—review and editing, R.Q., D.A, and Z.A.; review and editing Z.A and H.S.; writing—original draft preparation, R.Q; project administration, R.Q and Z.A.; data curation, H.T, and R.A.Competing interests: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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Establishment of CD4 and CD8 Lymphocyte subsets in a healthy HIV and Toxoplasma seronegative pregnant women in Libya

RESEARCH

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A B S T R A C T

Most of the diagnostic laboratories in Libya often depend on western textbooks for CD4+- and CD8+ T-lymphocyte reference values. In this paper, we established reference ranges for the Libyan *Toxoplasma*, *HIV, HBV, and HCV* seronegative healthy pregnant women in all trimesters of pregnancy, and compared them with a control group of non-pregnant women. Whole-blood samples were collected to provide normal ranges for CD4+ and CD8+ Lymphocyte subsets expressed as mean ± standard division. A total of 110 Libyan women who came from Tripoli and Zwara districts were investigated; 70 pregnant women (aged 27.8 ± 2.99, range 18-40 years old) and 40 non-pregnant women (aged 22.7±3.01, range 18-40 years old) were included as controls. All cases/controls were seronegative for toxoplasmosis, *HIV, HBV and HCV.* The CD4+ cell counts were 685±256 cell/µl at the first trimester (T1), 740±202 at T2, and 923±203 cell/µl at T3. While the CD8+ cell counts were 451±171 cell/µl at T1, 541±168 at T2, and 753±190 cell/µl at T3. The CD4:CD8 ratios were 1.5±0.64 at T1, 1.4±0.51 at T2, and 1.2±0.36 at T3. Moreover, the mean absolute CD4+ and CD8+ counts for the control group were 1001±232 cell/µl and 717±159 cell/µl respectively.

Absolute counts of CD4+ and CD8+ cells in pregnant women were significantly lower as compared to controls ($P<0.05$). Statistically significant decrease in the CD4+ and CD8+ cell counts was reported during T1 ($P<0.05$). These values increased significantly during the T2, and was comparable to the controls during T3 ($P>0.05$). The absolute CD4+ and CD8+ cell counts decreased with age for both groups. Geographical variation was reported for the cell counts between Tripoli and Zwara district at T3. We established reference ranges of CD4+ and CD8+ T-lymphocytes for the Libyan healthy pregnant women and discussed their use as prognostic markers. Further cohorts with greater sample size may be required to define the stage of the disease in relation to the normal CD4+ and CD8+ T lymphocyte count subsets in the Libyan population.

Keywords: CD4+-, CD8+ T-lymphocyte counts, Flow Cytometry, Libya, pregnancy, HIV, toxoplasmosis.

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Introduction

Toxoplasmosis, caused by a protozoan intracellular parasite *Toxoplasma gondii*, is one of the most common parasitic infections among humans and other warm-blooded animals (Montoya and Liesenfeld 2004). The

global seroprevalence of *T. gondii* in the general population varies widely and ranges between 10 and 70% (Montoya and Liesenfeld 2004, Xiao, Yin et al. 2010, Sun, Lu et al. 2013). Most cases are asymptomatic and the clinical disease is generally not recognized. However, complications may occur in immunocompromised individuals including AIDS patients, transplant recipients, and patients undergoing cancer treatment (Liu, Wang et al. 2015). These complications can range from neuropsychiatric disorders to severe and potentially fatal encephalitis due to the reactivation of latent infections in AIDS patients (Halonen and Weiss 2013).

T. gondii can pass through the placental barrier and infect the fetus (Jones, Lopez et al. 2003, Montoya and Remington 2008). Primary infections of pregnant women are associated with potential congenital infections and spontaneous abortions. The severity of congenital infections and frequency of transmission are influenced by the stage of pregnancy in which maternal toxoplasmosis is acquired (Halonen and Weiss 2013).

Transmission is relatively low (< 20%) during the first trimester resulting in spontaneous abortion, hydrocephaly and mental retardation. However, it increases up to 80% by the end of the pregnancy (Jones, Lopez et al. 2003, Ortiz-Alegria, Caballero-Ortega et al. 2010). The majority of the later cases are subclinical and resulting in asymptomatic infections or recurrent chorioretinitis that can lead to vision problems and potentially blindness (Montoya and Liesenfeld 2004).

The overall incidence of congenital toxoplasmosis in most populations, based upon serological methods, is from 1 in 1000 to 1 in 10,000 live births (Tenter, Heckeroth et al. 2000, Dubey and Jones 2008). However, a study measuring vertical transmission rates in humans using PCR detection of the umbilical cord found transmission rates of 19.8% (Hide, Gerwash et al. 2007). This suggests that human vertical transmission of Toxoplasma may be under reported when measured by serological methods (Hide, Morley et al. 2009).

Pregnancy is considered as physiological immunosuppression condition and can be associated with suppression of several immunological functions in order to accommodate the fetus (Dayama, Pandit et al. 2003). This includes the cell-mediated arm of the immune system which is conferred by the lymphocytes. These lymphocytes arise from hematopoietic stem cells in the bone marrow and have two major types: the B-cells, which when activated, differentiate into plasma cells and they secrete specific antibodies; and the T-cells, which rise in the thymus and have two main types: the first type differentiates on activation into cytotoxic T-cells, which directly kill cells infected with viruses, whereas the second type differentiates into cells that activate other cells such as B-cells and macrophages. The later includes the helper T- lymphocytes which assist the B-cells in antibody response and express the cluster determinant 4 (CD4+) molecules, while the cytotoxic T-cells express cluster determinant 8 (CD8+) molecules (Janeway Jr, Travers et al. 2001).

In several countries, prenatal screening of women is performed with the goals of early diagnosis and treatment of *T. gondii* infections (Roberts, Hedman et al. 2001). Moreover, serological diagnosis is routinely used to determine the immune status with regard to *T. gondii* infection (Jenum and Stray-Pedersen 1998). Estimation of CD4+ and CD8+ T-lymphocytes counts are used to measure the strength of an individual's immune response and continues to be an important aspect for monitoring of immune function (Yang, Wu et al. 2015). They are also used as indicators to begin prophylactic therapy against opportunistic infections (Force 1994). Other parameters that can be used to monitor the immune status include CD4+/CD8+ ratio, CD4+ and CD8+ percentages (Dayama, Pandit et al. 2003).

Available evidences suggest that the variations in CD4+ and CD8+T-lymphocytes could depend on certain important factors namely environment, ethnicity, genetic differences and dietary patterns in addition to age and gender (Tollerud, Clark et al. 1989, Lebranchu, Thibault et

al. 1991, Tsegaye, Messele et al. 1999, Fahey, Schnelle et al. 2000, Uppal, Verma et al. 2003). Moreover, CD4+ and CD8+T-lymphocytes counts are influenced by viral infections such as HIV, hepatitis B virus (HBV) and hepatitis C virus (HCV) (Zhou, Zhang et al. 2014), and parasitic infections such as toxoplasmosis which is highly prevalent in Libya. Seroprevalence among Libyan pregnant women ranges between 44.8% and 50% (Kassem and Morsy 1991, Magrhi, Abudher et al. 2003, Mousa, Mohammad et al. 2011). Moreover, 17.6% of women who suffered from spontaneous apportion in Tripoli were *Toxoplasma* seropositive (Gashout, Lazrag et al. 2008), and the prevalence of congenital toxoplasmosis was found to be 44% (Alkhunfas 2008). The diagnosis of *Toxoplasma* is based on serology. Recent introduction of molecular detection of the parasite was reported in Tripoli (Gashout, Amro et al. 2016). The development of monoclonal antibodies and flow cytometry technology has made possible new approaches to leucocytes subset identification. However, no studies on T-lymphocyte subsets were conducted and no country-specific immunohaematological reference values are available in Libya. Most of the diagnostic laboratories often depend on western text books for CD4+- and CD8+ T-lymphocyte reference values, therefore interpretation of these values in pregnant women is inappropriately based on reference values established for healthy nonpregnant women. Hence, we conducted this study to established reference ranges for the Libyan healthy Toxoplasma and *HIV*, *HBV*, and *HCV* seronegative pregnant women in all trimesters of pregnancy and compared them with a non-pregnant group before using these levels as prognostic marker.

Methods

Study settings and samples

Between 2008-2009, 70 pregnant women attended to antenatal Rahama clinic were selected for this study. Control group of 40 non-pregnant women were included for comparison. Women with a history of any disease (e.g., complications in pregnancy, cough, cold or fever in the past one month) were excluded.

Serologic tests

The ELISA test was used to assess Toxoplasmosis sero negativity for both groups. Approximately 5 ml of blood were collected from each subject. Serum was separated from the whole blood by centrifugation at 3000 rpm for 5 min and screened for anti-*Toxoplasma* IgG and IgM antibodies by using standard ELISA commercial kits (Human Gesellschaft für Biochemica und Diagnostica GmbH, Wiesbaden, Germany) in accordance with the manufacturer's instruction. All samples were analyzed in the Libyan National Centre for Infectious Diseases Prevention and Control (LNCDC). Moreover, all serum samples were tested for *HIV* (anti-*HIV* Tetra ELISA, Biotest Co. Dreieich, Germany), *HBV* hepatitis B antibody (Anti-HBc EIA WELL, Radim, Italy), and *HCV* antibody (HCV-Ab ELISA, DRG Co., Marburg, Germany),

Flow cytometry Immunophenotyping

To provide normal ranges for CD4 and CD8 Lymphocyte subsets, whole-blood samples (1 ml) were collected using sterile EDTA Vacutainer tubes. Three EDTA blood samples were collected from each pregnant woman and the test was performed every trimester of pregnancy. Samples were collected in triplicates between 9:00 -12:00 AM to avoid any error in subset counts due to diurnal variations (Carmichael and Abayomi 2006). Immunocytometry was done using CyFlow Counter machine (Partec, Münster, Germany) with two monoclonal antibodies; CD4 Antibody (MEM-241) [Phycoerythrin] and CD8 Antibody (MEM-31) [Phycoerythrin]. In brief, 20 µl of whole blood in EDTA was mixed with 20 µl of CD4 mAb PE or CD8 mAb PE and incubated for 15 min at room temperature protected from light. Red blood cells were then lysed by adding 800 µl of fluorescence-activated cell sorter lysing solution. After vortex, tubes were incubated in the dark at room temperature for 15 min then measured. The CA3 software provides instrument control, data

acquisition, data analysis, and True Volumetric Absolute Counting (Greve, Cassens et al. 2003).

Statistical Analysis

Measurement data among groups were expressed as mean ± standard division. Statistical analysis was carried out using GraphPad Prism version 5 for Windows. Comparison of the means was carried out using the student–t test. AP < 0.05 indicated that the difference was significant.

Results

A total of 110 Libyan women were investigated and divided into two groups, 70 pregnant women (aged 27.8 ± 2.99, range 18-40 years old) and 40 non-pregnant women (aged 22.7±3.01, range 18-40 years old) included as controls. All cases/controls came from Tripoli district except 10 pregnant women who came from Zwara district. To ensure that all cases/controls are free from infections, participants were screened for anti-Toxoplasma IgG and IgM antibodies and were seronegative. Moreover, all cases/controls were seronegative for, *HIV*, *HBV* and *HCV*.

Immunophenotyping of Peripheral Blood lymphocytes

The mean absolute CD4+ and CD8+ cells count ± SD and their ranges were calculated and compared for the two groups. Table 1 summarizes the results of the pregnant women group during the three trimesters of pregnancy. The CD4+ cell counts range from 685±256 cell/µl at the first trimester (T1) and 923±203 cell/µl at T3. While the CD8+ ranges from 451±171 cell/µl at T1 and 753±190 cell/µl at T3. The CD4:CD8 ratio ranges from 1.5±0.64 at T1 and 1.2±0.36 at T3 (Table 1). Moreover, the mean absolute CD4+ and CD8+ counts ± SD for the control group (Non-pregnant women) were found to be 1001±232 cell/µl and 717±159 cell/µl respectively (Table 1). Absolute counts of CD4+ and CD8+ cells in pregnant women were significantly lower as compared to non-pregnant group (*P*<0.05). A wide variation of CD4+ and CD8+ counts was reported between both groups (Table 1). During the first trimester of pregnancy, there were statistically significant decrease in the CD4+ and CD8+ cell counts compared to the control group (P<0.05). These values increased significantly during the T2 compared to T1. At the third trimester, CD4+ and CD8+ cell count increased to 923±203 and 753±190 which were comparable to the values of the control group and did not show statistically significant differences (*P > 0.05*) (Table 1).

Table 1. CD4 and CD8 cell counts and ratios for cases and controls [mean ± SD (range)]

Counts	Pregnant women (n = 70) (study group) [Data are mean ± SD (range)]			Non-pregnant women (n= 40) control group
	1st Trimes.	2nd Trimes.	3rd Trimes.	
CD4+ (count/µl)	*685±256 (182-1639)	*740±202 (209-1458)	923±203 (214-1720)	1001±232 (420-1718)
CD8+ (count/µl)	*451±171 (126-924)	*541±168 (144-907)	753±190 (150-2008)	717±159 (310-966)
CD4:CD8 ratio	1.5±0.64 (0.19-3.13)	1.4±0.51 (0.16-3.38)	*1.2±0.36 (0.22-2.4)	1.4±0.25 (1.07-2.14)

* *P* < 0.05 compared to control

There was no statistically significant change in the CD4:CD8 ratio during the first and second trimesters, however, it decreased significantly from 1.5 at T1 and 1.4 at T2 to 1.2 at T3 (P<0.05). Moreover, significant decrease of CD4:CD8 was seen between T3 and control group (Table 1). Further analysis of our results showed that the absolute numbers of the CD4+ and CD8+ T-lymphocyte cell counts decreased with age for both groups except the CD4+ cell counts for the pregnant group (Table 2).

T-lymphocyte subsets values were compared between Tripoli and Zwara districts. Significant increase in CD8+-cell counts was predicted in Zwara (869± 50 cell/µl) compared to Tripoli district (722± 61 cell/µl) only at the 3rd

Table 2. Age distribution of CD4 and CD8 subset values among pregnant and non-pregnant Libyan women.

Age group	Women Pregnant			Non Pregnant women		
	Number of cases	CD4	CD8	Number of cases	CD4	CD8
18-22	5	163±602	250±590	5	1138±379	769±177
23 – 27	29	216±863	157±623	15	1022±138	770±119
28 –32	21	139±684	118±508	12	1013±160	739±127
33 – 37	12	206±848	174±645	6	999±297	644±179
38 - 40	3	142±730	63±449	2	754±86	508±7
Total	70			40		

significant differences in CD4+-cell counts were found between the two districts for pregnant and non-pregnant groups.

Discussion

In this study, we established reference ranges of CD4+ and CD8+ T-lymphocytes for the Libyan healthy, HIV, HBV, HCV, and Toxoplasmosis seronegative pregnant women and discussed their use as prognostic markers. The absolute CD4+ and CD8+ cell counts in pregnant were significantly lower than non-pregnant women. This is consistent with the findings of most of studies that examined the effect of pregnancy on T-lymphocytes counts. Clinical data on pregnant women shows a deviation of the immune system consistent with a weakening of the cell-mediated immunity and strengthening of humeral immunity (Wegmann, Lin et al. 1993), which is important for the success of pregnancy (McIntire and Hunt 2005, Aagaard-Tillery, Silver et al. 2006, Hunt 2006, Munoz-Suano, Hamilton et al. 2011).

An Indian study among HIV-negative people found absolute CD4+ cell counts to be significantly lower in pregnant than in non-pregnant women (Dayama, Pandit et al. 2003). An earlier study among Africans demonstrated reduced absolute values of CD4+, CD8+ and total lymphocytes during pregnancy (Vassiliadou and Bulmer 1998). However, the mean absolute values of CD4+, CD8+ counts in our study were higher than those reported from Nigerian (Aina, Dadik et al. 2005) and Chinese reports (Jiang, Kang et al. 2004) and were comparable to the Indian reports (Murugavel, Balakrishnan et al. 2009). The relationship between gestational age and T-lymphocyte levels in pregnant women varies in the literature. A study in Kenya found no relationship between gestational age and any immunological variable in both HIV-positive and negative women (Temmerman, Nagelkerke et al. 1995). Ibitokou et al. found significant decrease of CD4+ cell counts between the second trimester and delivery of the sub-Sahara African women (Ibitokou, Brutus et al. 2013). Conversely, Tuomala et al., found an increase in CD4+ cell counts of 2.76 cells/uL per week of pregnancy during serial measurements in pregnant women (Tuomala, Kalish et al. 1997). In our study, significant decrease of CD4+ and CD8+ cell counts were predicted during the first trimester followed by significant increase during the second trimester, and were comparable to the control group in the third trimester. These discrepancies could be related to the significant geographical and racial differences described in earlier studies. For example, CD4+-cell counts for Asian was found to be lower than that recorded for Caucasians (Lee, Yap et al. 1996), and among African populations, healthy Ethiopians (Tsegaye, Messele et al. 1999) have markedly lower counts

than those recorded in Uganda (Tugume, Piwowar et al. 1995) and Tanzania (Levin, Brubaker et al. 1996). Hence, this establishment of country-specific immunehaematological reference values in Libya is crucial.

The CD4:CD8 ratio was comparable to the control group during T1 and T2. This indicates uniform decrease of the tow lymphocytes subsets during these periods. However, the significant decrease of the ratio during T3 indicating faster increase of CD8 compared to that for CD4 cells during this period.

The CD4+ and CD8+ T-lymphocyte cell counts decreased with age for both groups except the CD4+ cell counts for the pregnant group. This is in agreement with a previous study concluded that maternal age had no significant effects on CD4+ cell count levels in pregnancy (Akinbami, Gbadegesin et al. 2015), and disagrees with the results from Abimiku et al. which reported that low CD4+ cell count was significantly associated with older age(Abimiku, Villalba-Diebold et al. 2009). Moreover, Lugada et al reported that CD4+ cell count is highest during the early years of life, declines steadily to stable adult values, and it is lowest in the elderly (Lugada, Mermin et al. 2004). However, our results are limited with smaller sample and need to be validated with larger cohorts.

Geographical variation in the CD8+-cell counts has been reported through Zwara district compared to Tripoli district at the 3rd trimester of pregnancy. Though our sample size is very small, these results emphasize the need for further investigation on T-lymphocyte counts variation between different districts in Libya. There are many factors affecting the levels of circulating lymphocytes and may lead to differences across regions. This includes genetic makeup, altitude, dietary patterns, body mass index and smoking habits (Schaberg, Theilacker et al. 1997, Feldman, Minkoff et al. 2006, Mair, Hawes et al. 2008).The influence of these factors point out to the fact that the T-lymphocytes reference ranges of one population might not be precisely used as a reference range for another, and might give an inaccurate interpretation of the immune status.

The findings of the present study though important, are limited with small sample size and restricted to two districts and hence cannot be generalized. Another limitation is that the samples were collected during 2008-2009 and that current values can vary according to environmental and nutritional conditions. Nevertheless, our study design was unique since we evaluated the T-lymphocytes counts during the three trimesters, while most of studies in the literature were conducted only on the first trimester of pregnancy.

The lymphocytes reference values have been established by many studies throughout the world and have shown some variability according to geographical locations and methodology (Bosire, Nyamache et al. 2013). The validity of comparison of CD4+ and CD8+ cell counts depend on the comparability techniques, duration and temperature of sample storage which could differ significantly between studies.

Moreover, assay variations may be attributed largely to processing methods, monoclonal antibodies, analysis methods and lag period between drawing blood and processing of the specimen (Landay and Muirhead 1989). Hence, the use of single – platform flow cytometry, and quality control of this platform are recommended to eliminate some of the variability between different studies and making them more comparable. This platform should be used for future studies in order to ensure correct comparability and interpretation of the results (Chng, Tan et al. 2004).

Conclusions

In conclusion, the establishment of normal ranges for T-lymphocytes with the local population is a helpful tool to clinicians for the better clinical management of pregnancy specific-diseases in Tripoli and other surrounding areas, and they are used for clinical classification, to determine prognosis, and to decide whether to prescribe prophylaxis for opportunistic infections especially Toxoplasmosis which is highly prevalent in Libya. Further cohorts with greater

sample size may be required to define the stage of the disease in relation to the normal CD4+ and CD8+ T lymphocyte count subsets in the Libyan population.

Declarations

Ethics approval and consent to participate

All aspects of the study were revised and approved by the ethics committee of the Libyan National Centre for Disease and Control (LNCDC). Prior to starting the study, study objectives and procedures were explained for each participant. Informed written consent was obtained from all participants. Confidentiality was ensured through secure data management, and no personal identifiers were in the computer system. Data and samples were labelled with anonymous identification numbers. Test results were confidentially disclosed to the subjects following post-test counselling.

Consent for publication

Not applicable

Availability of data and material

All data generated or analysed during this study are included in this published article

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

AG and AA designed the study, AG, HAD, ME, AAl, and AAb investigated the patient and have done the clinical evaluation and laboratory tests. AG and AA analysed the data. AG and AA have written the manuscript. All authors have read the manuscript and approved its content

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Epidemiology of Enterobiasis in Palestine

RESEARCH

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A B S T R A C T

Enteropiasis is a parasitic disease caused by the pinworm; *Enterobius vermicularis*. In this report, the prevalence of *Enterobius vermicularis* infection in the West Bank and Gaza strip was investigated based on Palestinian Ministry of Health reports from 2008 to 2018. A total of 29,390 cases was reported, 29,061 (98.9%) in the West Bank, and 329 cases (1.1%) in Gaza Strip. The results of the present study show that *E. vermicularis* infection is highly prevalent among people living in the West Bank and to lesser extend in Gaza Strip. There is a need for joint and concentrated efforts from the Palestinian government and public health services to control this infection. Personal hygiene, education and living conditions and overcrowding are risk factors associated with the spread of infection.

Keywords: Enterobius vermicularis, Palestine, infectious diseases, West Bank, Gaza

Introduction

Parasitic infections are among the common contagious diseases of the Palestinian community (Hamarsheh and Amro 2020), previous reports documented the widespread of certain parasitic infections like Leishmaniasis (Amro and Hamarsheh 2020), scabies (Hamarsheh 2020, Amro and Hamarsheh 2012), intestinal parasites (Astal 2004, Mezeid et al. 2014, Al-Hindi 2002, al-Agha and Teodorescu 2000).

Enterobiasis is a cosmopolitan parasitosis caused by the pinworm *Enterobius vermicularis* (*E. vermicularis*). Direct contact between infected individuals with others are considered the main rout of transmission (Sato et al. 2008, Al-Hindi 2002, Stoyanova et al. 2020). Many studies have been conducted to explore the risk factors associated with enterobiasis disease. The main risk factors are associated with indoor living conditions, including personal sanitary and close contact with other people, as pinworms spread mainly indoors directly from one human to another (Abu Mourad 2004, Acosta, et al. 2002, Kim et al. 2010, Song et al. 2003).

Crowded communities like refugee camps and overcrowded regions are optimal for the transmission and spread of enterobiasis. Kindergartens, nurseries and primary schools are places with high transmission risks (Hussein 2011, Remm 2006). The disease is not fatal and may remain asymptomatic or cause perianal pruritus, insomnia, restlessness, irritability, and rarely, impetigo of scratched skin, vulvovaginitis, or enuresis. Although effective medications have been available for decades, control of enterobiasis has been difficult because of

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reinfection, incomplete cure of infected people, and its ready transmissibility. The symptoms of enterobiasis are not fatal, and it can be readily transmitted via direct contact between infected and uninfected persons. Although the majority of infections are asymptomatic, they can induce bothersome symptoms in some cases, including an itching sensation and irritation in the perianal area, along with mental distraction. The disease is asymptomatic in adults, however, in children, particularly those with high worm burdens, restlessness, irritability, and distraction may occur, and these may influence child growth (Karamitros et al. 2017, Carrillo-Quintero et al. 2016, Fedotova 1999). *E. vermicularis* infection is prevalent in many countries of the world, including developed countries. *E. vermicularis* infection is diagnosed among school children in Palestine and according to the Ministry of Health annual reports, the average prevalence of the infection reaches very high levels (Hamarsheh and Amro 2020). There are many studies to explore the epidemiology of parasitic diseases in Palestine (Hamarsheh and Amro 2020, Hindi 2014, Kanoa and Al-Hindi 2009, Astal 2004, Abu Mourad 2004). Currently, little information is available regarding the prevalence and the risk factors of enterobiasis. Therefore, this report aims to investigate status and prevalence of enteropiasis disease in the West Bank and Gaza strip.

Methods

Study areas

The study were carried out in the West Bank and Gaza Strip. These geographical areas are unconnected and almost separated. The West Bank consists of the following districts: Hebron, South Hebron, Bethlehem, Jerusalem, Ramallah, Jericho, Nablus, Jenin, Tubas, Tulkarem, Qalqilya, and Salfit. The Gaza Strip has the following districts: North Gaza, Gaza City, Deir Al Balah, Khan Younis, and Rafah (see Figure 1). The total Palestinian population in 2017 was 4.95 million (three millions in the West Bank and 1.95 million in Gaza Strip). About 66.2% of the Palestinian population residing in the Gaza Strip and most of the people there live in overcrowded areas, on the other hand 26.6% residing in the West Bank with more space and open areas, even though there are number of refugee camps in the West Bank.

Data collection and analysis

Since enteropiasis disease is considered one of the reportable parasitic diseases, the Palestinian Ministry of Health annual statistical reports were screened for the years 2008 until 2018 (the latest published report). For data analysis detailed epidemiological information about the diseases, and the number of patients per year in each Palestinian district was determined and arranged in spread sheet.

Results

Enteropiasis Disease is existed in most Palestinian districts of the West Bank and in all districts in Gaza Strip (Figure 1).

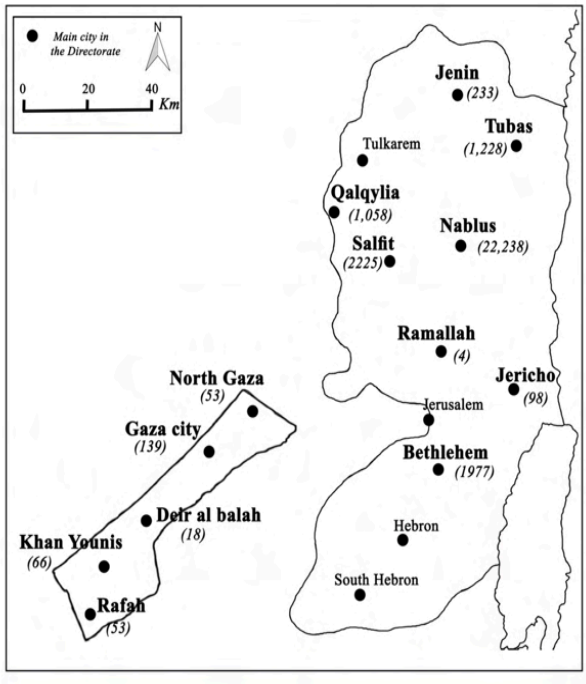

Figure 1: Map of the West Bank and Gaza Strip, Palestine shows the distribution of enteropiasis disease in the country from 2008-2018

The total number of cases in Palestine from 2008 to 2018 was 29,390 cases, among them; 29,061 (98.9%) in the West Bank, while the others

329 cases (1.1%) in Gaza Strip. Nablus district was found to have the highest number of cases (75.7%) followed by Salfit (7.6%), Bethlehem (6.7%), Tubas (4.2%), and Qalqylieh (3.6%). The other districts; Hebron, South Hebron, and Jerusalem were totally free of the disease or have very low numbers; Jenin (0.8%), Jericho (0.3%), and Ramallah (0.01%). The distribution of cases per year and district are shown in Table 1. The yearly trend of the disease, as shown in Figure 2 is relatively steady with slight fluctuations, the highest peak (13740 cases), and the lowest peak was in 2011 where only 624 cases. There is a peak in 2010 and 2013 with 1748 and 2595 cases reported respectively. From 2016 and ahead there is slight change on the number of reported cases every year.

Table 1: Total number of Enterobiasis patients from 2008-2018 in each directorate.

District	2008	2009	2010	2011	2012	2013	2014	2015	2016	2017	2018	Total
N.Gaza	1	5	2	3	7	19	8	2		6		53
Gaza City	3	5	33	49	8	11	2	15	5	7	1	139
Deir Al-Balah	1	1	1	2	5	1		4			3	18
Khan Younis			25	4	3	5	26				3	66
Rafah	3	11	6	7	8	3	8	4		2	1	53
Sum (Gaza Strip)	8	22	67	65	31	39	44	25	5	15	8	329
Hebron												0
S.Hebron												0
Bethlehem	62	198	113	91	252	340	253	267	170	97	134	1977
Jerusalem												0
Ramallah											4	4
Jericho	2	20	1	2	21	7	11	25	4	1	4	98
Nablus	13424	1342	1371	416	1210	1430	1199	503	469	414	460	22238
Jenin								213	9	9	2	233
Tulkarem												0
Qalqylieh	25	135	36	10	19	98	109	113	114	167	232	1058
Salfit	219	243	160	40		358	315	224	206	211	249	2225
Tubas					157	323	246	213	149	88	52	1228
Sum (West Bank)	13732	1938	1681	559	1659	2556	2133	1558	1121	987	1137	29061
Total Palestine	13740	1960	1748	624	1690	2595	2177	1583	1126	1002	1145	29390

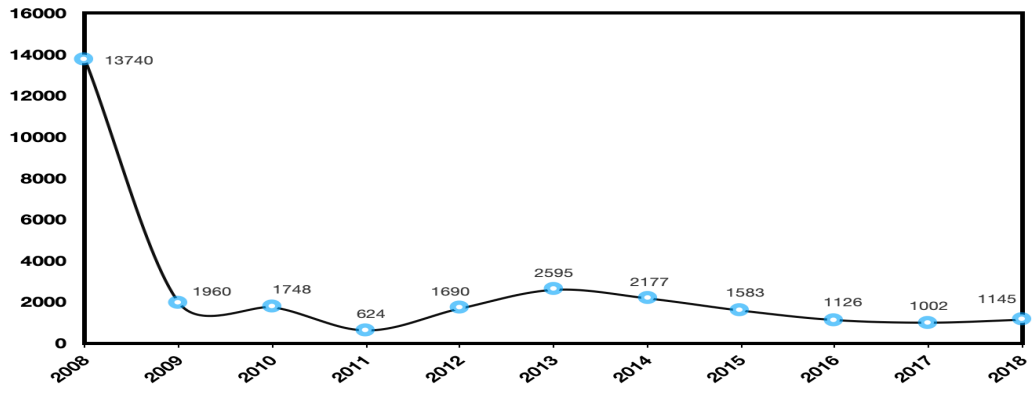

Figure 2:

The yearly trends of enterobiasis from 2008 to 2018 as total number of cases per year.

Discussion

The infection rate of entorobiasis was compared between directorates in the West Bank and Gaza Strip from 2008 to 2018. The disease was prevalent in the West Bank (98.9%), the majority of cases are distributed in certain directorates; the disease is highly endemic in Nablus and this is not surprising since this directorate is considered highly populated and have many overcrowded refugee camps, other parasitic diseases were reported previously in this directorate (Hamarsheh and Amro 2020). Tulkarem, Hebron, Jerusalem and South Hebron directorates in the West Bank are totally free of the infection. Low numbers in Hebron district and Jerusalem is probably due to problems in the reporting system since most of Jerusalem area is under Israeli control and enteropiasis cases are counted as Israeli cases. On the other hand, very few cases reported in Ramallah directorate (4 cases in 2018 only), and in Jenin (233 cases) from 2015 to 2018. Distribution of the infection in Gaza strip is considered very low compared with the distribution of the disease in the West Bank directorates (329 cases in Gaza Strip in 10 years). The unequal distribution of the eneteropiasis cases in the Palestinian directorates should be subjected for further research, overcrowdings seems not the only factor that support and maintain the infection in the country. Variations between inhabitants in socioeconomic levels, personal hygiene, nutrition, education, and living conditions are important factors that may have a role in the spread of the disease.

The high infectivity of *E. vermicularis* is due to multiple transmission modes (anus to mouth, food, dust, retrograde from anus to intestine) and prolonged egg viability (14 days). This is different from other helminths, it can reproduce in humans without passing through an intermediary soil phase. Thus, it can be transmitted from person to person. In rural or overcrowded areas enterobiasis transmission is facilitated by difficulties in maintaining good personal hygiene. These living conditions are available in Palestine. Although enterobiasis disease is not fatal, but considered to be a nuisance. The level of morbidity is significant, particularly in children. Therefore, this worm is one of the most frequently encountered and ubiquitous nematodes. Although enterobiasis can be readily cured by anthelmintic medications, the prevalence of this infection in Palestine has not significantly diminished yet. Personal hygiene is closely associated with *E. vermicularis* infection, inadequate personal hygiene can increase the risk of enterobiasis among primary school children. Other factors that significantly associated with enterobiasis include playing on the floor, nail biting, failure to wash hands before meals, inappropriate living conditions in marginalized or rural poor dwellings. Further research is crucial to identify environmental risk factors associated with enterobiasis especially in nurseries and kindergartens.

In conclusion, *E. vermicularis* infection was widely prevalent in some directorates in the West Bank. Therefore, raising awareness and systematic control and preventive programs for children at schools should be implemented in order to eradicate *E. vermicularis* infection.

Acknowledgements

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Conflict of interest

The author declare no conflict of interest in relation with this manuscript.

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Characterization of *Leishmania* Ulcers Microbiota Using Next Generation Sequencing

RESEARCH

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A B S T R A C T

The human skin microbiome is a major source of bacteria in cutaneous leishmaniasis (CL) ulcers following the fall of the crust and the subsequent formation of a shallow depression in the epidermis and dermis of the skin. As a result, secondary bacterial infections are frequently observed which impair the healing process. Our study aimed to investigate the bacterial communities in CL lesions using next-generation sequencing. A total of 298 patients (178 males and 120 females; the median age of 17) presenting ulcerated skin lesions suspected with CL were included in this study. CL was confirmed in 153 (51%) cases by ITS1-PCR and/ or microscopy. Based on bacterial 16S rRNA-PCR, 92 samples were positive for the presence of bacteria, while 206 samples were negative and excluded from the microbiome study. A total of 925 Operational Taxonomic Units (OTUs) were identified and assigned to 215 genera. Despite an insignificant difference in the microbiome composition between CL and non-CL lesions, the phylum level analysis revealed that *Actinobacteria* was significantly higher in CL ulcers while *Proteobacteria* was significantly higher in non-CL ulcers (X^2, P=0.039). The relative abundance of the most commonly encountered skin pathogens i. e *E. coli*, *Pseudomonas aeruginosa*, *Enterobacter*, *Enterococcus* and *Acinetobacter* species were significantly higher in non-CL ulcers (X^2, P<0.05) compared to *Staphylococcus aureus* and *Proteus mirabilis* which was higher in CL ulcers (P<0.05). Our data showed that bacterial communities did not cluster according to the *Leishmania* infection. Nonetheless, bacterial diversity was lower in CL compared to non-CL lesions. Presence of pathogenic bacteria in CL lesions such as *S. aureus* might exacerbate lesions, hinder diagnosis, and delay healing.

Keywords: Leishmaniasis, CL lesion, Microbiome, Next-generation sequencing.

Introduction

Cutaneous leishmaniasis (CL) is the most common form of leishmaniasis, occurring either as zoonotic or anthroponotic infections caused by different species of the genus *Leishmania*. In the Middle East and North Africa, zoonontic CL is mostly caused by *Leishmania major* while anthroponotic CL is caused by *L. tropica* (Tabbabi, 2019). It is transmitted following a bite from an infected female sandfly, *Phlebotomus* spp. After an incubation period of several weeks to several months, CL manifests itself starting from small erythematous papules through nodules to ulcerative lesions which later develop crusts that fall after a period time leaving a crater-like depression(Ashford, 2000; Klaus and Frankenburg, 1999; Yanik, Gurel et al., 2004). The healing or chronicity of the CL ulcer is essentially dependent on skin integrity and immune system. Shortly after sandfly inoculation, infective promastigotes evade host innate immunity to survive. The ongoing battle between the host immune response and the invading parasites will decide the fate of the disease. It is well known that T cells play an essential role in the resolution of infection. Thus, individuals with a T cell response, characterized

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by delayed-type hypersensitivity (DTH) and high levels of interferon λ (IFN-λ), control the parasite load in their lesion compared to those with only a humoral response (Al-Jawabreh et al., 2017; Scott and Novais, 2016). However, environmental exposure and poor hygiene at the lesion site may promote polymicrobial infections (Salgado, Queiroz et al., 2016). Moreover, *Leishmania* parasites normally disrupt the natural skin barrier to establish cutaneous lesions that predispose to bacterial infections and can further cause dysbiosis by changing the composition of skin microbiota (Gimblet, Meisel et al., 2017; Silva-Almeida, Pereira et al., 2012). Secondary bacterial infections are frequently observed in 22% to 68% of the CL patients (Fontes, Carvalho et al., 2005; Vera, Macedo et al., 2006). skin microbiota was limited to culture-dependent assays with several culture-based studies reporting *Staphylococcus* spp, *Streptococcus* spp, *Enterococcus* spp, *Pseudomonas* spp, *Escherichia coli*, *Proteus* spp, *Klebsiella* spp and other opportunistic bacteria in CL lesions (Vera, Macedo et al., 2006; Ziaei, Sadeghian et al., 2008). The secondary bacterial infection increases tissue destruction, prolongs the duration of CL lesion, exacerbate lesions, hinders diagnosis, decreases the efficacy of CL treatment, and raises the probability of scar formation (Sadeghian, Ziaei et al., 2011). The skin microbiome containing a huge abundance of microorganisms which is a major source of secondary infection with several factors being reported as having the ability to alter the composition of skin microbial community such as behavioral factors, hygiene practices and use of cosmetics (Casadevall and Pirofski, 2015; Qian et al., 2012; Shin, Pei et al., 2015). Recently, new molecular approaches revealed that only about 1-2% of the skin-colonizing bacteria could be cultivated under usual conditions indicating the insufficient sensitivity of culture-based methods (Bertesteanu et al., 2014). The 16S small subunit ribosomal (rRNA) gene is universal among prokaryotes and contains 9 hypervariable regions (V1-V9) of varying conservation. More conservative regions are helpful for determining the higher ranking taxa whereas more quickly evolving ones can be used for molecular identification of the majority of bacterial genera and species (Bukin et al., 2019). Based on this, microorganisms colonizing the skin fall into four different phyla: *Actinobacteria*, *Firmicutes*, *Bacteroidetes* and *Proteobacteria* (Weyrich et al., 2015). This study aimed to reveal the bacterial microbiome composition underlying CL ulcers and to investigate the relative abundance of different genera and species.

Methods

Subjects and samples collection

A total of 298 patients presenting ulcerated skin lesions clinically suspected with CL were recruited in this study. The dermal tissue scrapings were sampled from the border of one lesion, after extensive cleaning with 70 % alcohol (3 times) as previously described (Al-Jawabreh, Schnur et al., 2004). The sampling involved the outer surface layer (epidermis) and the deeper layer (dermis). All tissue scrapings were blotted on sterile filter papers for ITS1-PCRs and on slides for microscopy. The smear samples were prepared and stained with Giemsa's stain and then examined under the microscope (X100) (100x)for the presence of *Leishmania* amastigote forms. Patients were confirmed to be CL after positive results in any of the two tests, ITS1-PCR or microscopic examination.

DNA Extraction

Genomic DNA was extracted from each dried tissue spots on filter papers according to manufacturers' instructions (Purelink®, Invitrogen™, CA, USA). DNA samples were frozen at –80°C until use. All samples (CL and non-CL, n=298) were processed with the same DNA extraction method to minimize variation in DNA yield which may affects the relative abundance of the bacteria measured at genus or species level (Kennedy, Walker et al., 2014).

PCR-RFLP of the Internal Transcribed Spacer 1 (ITS1)

All samples were amplified targeting a fragment of 300 bp of the ITS1 gene using 400 nM of LITSR and L5.8S primers (Schonian, Nasereddin et al., 2003). The reaction was carried out with PCR-Ready Supreme mix (Syntezza Bioscience, Jerusalem) for a total reaction volume of 25µl. Amplification conditions were as described previously, in brief PCR amplification was done as followed: initial denaturation at 95°C for 2 min

followed by 34 cycles consisting of denaturation at 95°C for 20 sec, annealing at 53°C, and extension at 72°C for 1 min. This was followed by a final extension cycle at 72°C for 6 min (el Tai, Osman et al., 2000; Schonian, Nasereddin et al., 2003). The obtained PCR products were digested with Hae III enzyme, according to the manufacturer's instructions and visualized by UV light on 2% agarose gels by electrophoresis at 120 V in 1X Tris-acetate-EDTA buffer (0.04 M Tris acetate and 1 mM EDTA, pH 8).The 100 bp GeneRuler DNA ladder Mix (Fermentas, MBI) was used as the DNA molecular marker. Reference strains of *L. tropica* (MHOM/AZ/1974/SAF-K27), *L. major* (MHOM/TM/1973/5ASKH), and *L. infantum* (MHOM/TN/1980/IPT1) were used as positive controls.

16S rRNA library preparation and sequencing

The hypervariable V3-V4 region of the 16S rRNA gene from each sample was amplified as previously described using the degenerate primer set (forward primer: 5'TCGTCGGCAGCGTCAGATGTG TATAAGAGACAGCCTACGGGNGGCWGCAG, reverse pr imer:5'GTCTCGTGGGCTCGGAGATGTGTATAAGAGACA GGACTACHVGGGTATCTAATCC)(Caporaso, Lauber et al., 2012). Amplification reactions were performed using X2 KAPA HiFi HotStart Ready Mix (Kappa Biosystems) with a final volume of 25µl. The PCR conditions were as follows: 95°C for 3 minutes, followed by 25 cycles of 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds, then 72°C for 5 minutes and final hold at 4°C. Negative controls containing nuclease-free water were used in each PCR run. Five microliters (5µl) of the amplified product were loaded on 2% agarose gel to visualize a band of ~500bp and to confirm successful amplification. PCR products were purified using AMPure XP beads (X0.8) followed by a second round of amplification using the Nextera XT Index Kit (Illumina). The prepared libraries were normalized and then pooled at 10 nM concentrations, 5µl of 4 nM was denatured, and mixed with *PhiX* control (15%) and finally loaded onto an Illumina *MiSeq* machine using *Miseq* 500 cycle kit (Illumina). The reaction preparations and PCR conditions were described in the online 16S Metagenomic Sequencing Library Preparation guide (Illumina). The 16 rRNA gene raw sequence data were quality-filtered and analyzed using CLC Genomics Workbench 9.0 (Qiagen, Denmark).

Bioinformatics and Statistical analysis

Pairwise distances were calculated between all DNA sequence reads. Then, reads were clustered into operational taxonomic units (OTUs) at the 0.03 level, meaning that sequences that displayed >97% similarity with each other were considered the same OTU. The taxonomic affiliation of each OTU was based on the Ribosomal Database Project (RDP-II) database. The OTUs were arranged in a data matrix where each row was a single sample and each column a specific OTU; each data point in the matrix represented the abundance of the particular OTU in the particular sample, relativized to the sampling effort, i.e. the number of reads obtained from that sample(McMurdie and Holmes, 2014). All OTUs identified to species or genus level were included in the study, while those not reaching this level were filtered out. Relative abundance of bacteria at genus/species level was displayed in the form of heat map clustered and scaled by correlation distance and average linkage. The software Rstudio, clustVis (http://www.rstudio.com/) and XLSTAT were used for data analysis and representation.

Ethics

Since this study involved the analysis of samples obtained during routine diagnostic work, patients were not asked to give their informed consent. However, patient data were anonymized for all laboratory and data analyses. The study design was approved by the ethics committee of Al-Quds Public Health Society under permission number 184/2014.

Results

Study Subjects

A total of 298 patients (178 males and 120 females; the median age was 17; and mean age ±SD (standard deviation) was 20.98±1.04) presenting ulcerated skin lesions suspected with CL were recruited from 11 districts in Palestine from 2004 to 2016. Palestine is in the north-eastern hemisphere encompassed between the points 31° 19' 23.47'' N-34° 13' 08.78'' E in the west and 31°

45' 37.35'' N- 35° 33' 31.43'' E in the east as well as between the points 33° 17' 26.79'' N-35° 34' 05.91'' E in the north and 29° 29' 27.51'' N-34° 54' 13.54'' E in the south. The absolute minimum temperature was -2 C° recorded in January in the mountainous areas while the absolute maximum temperature was 48.2 C° recorded in August in the Jordan valley (Jericho). The number of collected samples per district was shown in Figure 1. On examination, 192 (64.4%) patients showed one lesion while 106 (35.6%) pa¬tients presented multiple lesions (ranged from 2 to 12) at different body sites. The duration of the lesions ranged from 1 week to 3 months.

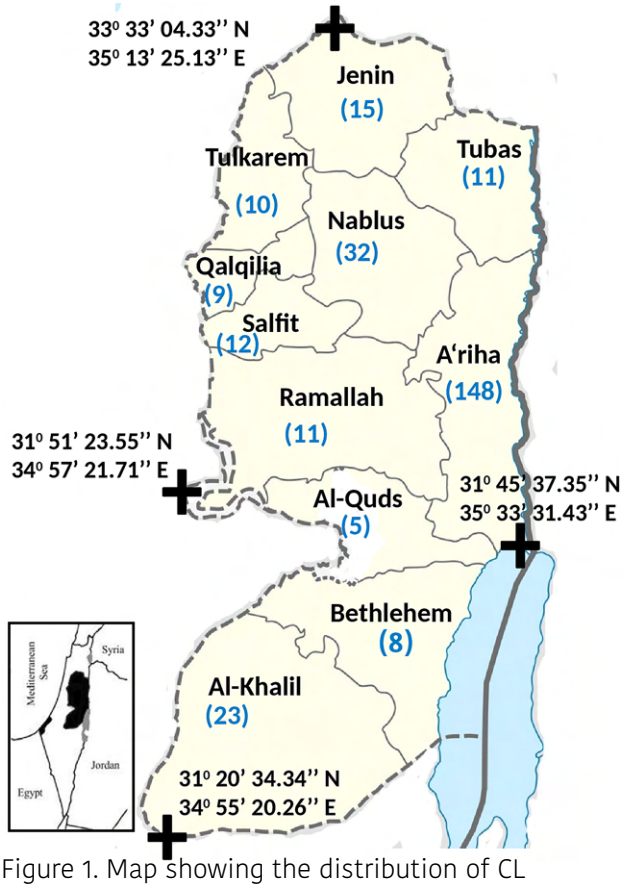

Figure 1. Map showing the distribution of CL suspected cases in the study area

Detection and identification of Leishmania parasites

All samples (n=298) were tested by ITS1- PCR and microscopic examination of Giemsa-stained smears. CL was diagnosed by demonstrating the amastigote stage of the *Leishmania* parasite or detecting DNA in tissue specimens spotted on filter papers. Out of the total samples, 153 (51%) of the cases were confirmed *Leishmania* positive at least by one method. On the other hand, 145 (49%) samples were negative by both tests and designated as non-CL lesions since the causative pathogen was unidentified. Of the ITS1-PCR positive samples, RFLP analysis showed 20.8 % as *L. major* and 71.8% as *L. tropica* (Figure 2).

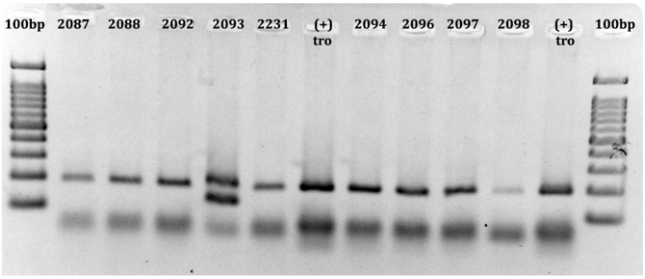

Figure 2: RFLP (Restriction fragment length polymorphism) pattern of ITS1 amplicon with *Hae III*. The patterns shown represent *L. major* (lane 5, Rec 2093) and *L. tropica* (rest of lanes) isolated from different patients in Palestine anonymized by record (Rec) numbers.

Analysis of 16S rRNA sequences

The hypervariable V3 and V4 regions of the bacterial 16S rRNA gene from all DNA samples (CL and non-CL, n=298) were amplified. Results showed that 28.7% (44/153) of CL lesions and 33.1% (48/145) of non-CL lesions samples were positive by16S rRNA–PCR. In total, 92 samples which showed bands indicating a positive result for 16S rRNA PCR were sequenced, while 206 samples were negative and, therefore, excluded from the microbiome study. Out of 92 samples, 27 (29%) were taken from patients who received antibiotics before time of sampling, 52 samples (56.5%) were from untreated patients and 13 (14%) were uncertain. After the quality filter checks, 2,218,116 high quality reads were obtained: 811,295 reads were from CL ulcers and 1,406,821 reads from non-CL (Table 1). The average length of the sequences was 442 bp. The OTUs were filtered down from a total of 1448 to 925, based on the genus level of identification; 896 OTUs in CL and 907 in non-CL ulcers (Table 1). All OTUs were assigned to 215 genera; 183 genera in CL and 197 in a non-CL group.

The 925 OTUs were, then, filtered down to 274 according to the likelihood of pathogenicity.

Table 1. Sequence analysis result profile

Parameter	CL lesion	Non-CL lesion	Total
No. of samples	153	149	298
No.16S rRNA-PCR positive	44	48	92
No.of treated cases prior to sampling	33	46	76
No. of reads	811,295	1,406,821	2,218,116
Number of genera	183	197	215
No. of OTUs	896	907	925

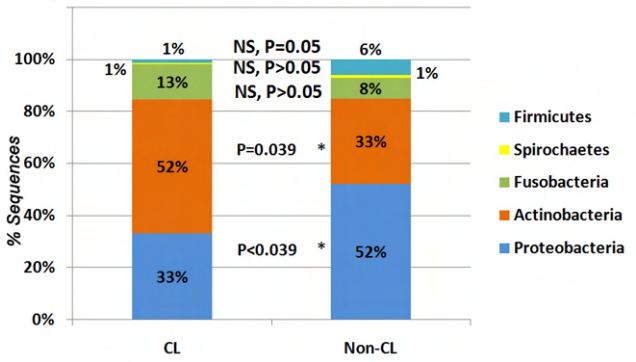

Figure 3. Diversity of potentially-pathogenic bacteria in CL ulcers vs non-CL ulcers. Colors of the stacked bar-graph represent the bacterial taxa at the phyluml evel in the 274 OTUs. NS: not significant.

The 274 OTUs were collapsed into their basic 79 species and genera. Using *R* studio software, the grand mean (mean of means) for the number of reads of CL patients was 16.8, while it was 35 for non-CL, showing a high difference in reads between the two groups (P=0.013, X^2). Most sequences detected in both groups were assigned to five core phyla: *Proteobacteria*, *Actinobacteria*, *Firmicutes*, *Fusobacteria*, and *spirochaetes* with different proportions (Figure 3). The Gram-negative Proteobacteria and the Gram-positive *Actinobacteria*, well-known skin microbiota (Cosseau, Romano-Bertrand et al., 2016), represent the main bacteria in both types of ulcers. Cumulative percentage of sequences from the bacterial phyla *Actinobacteria* were significantly higher in CL ulcers (P=0.039), while *Protoeobacteria* was significantly higher in non-CL ulcers (P=0.039). The 79 species/genera were further collapsed into 32 literature-reported skin and soft tissue pathogens in humans with varying degrees of pathogenicity, as some species were merged into their original genera (Chiller, Selkin et al., 2001; Ki and Rotstein, 2008; Moet, Jones et al., 2007). According to the bacterial pathogenicity, these genera/species were classified into two groups; most likely to cause disease and less likely, the

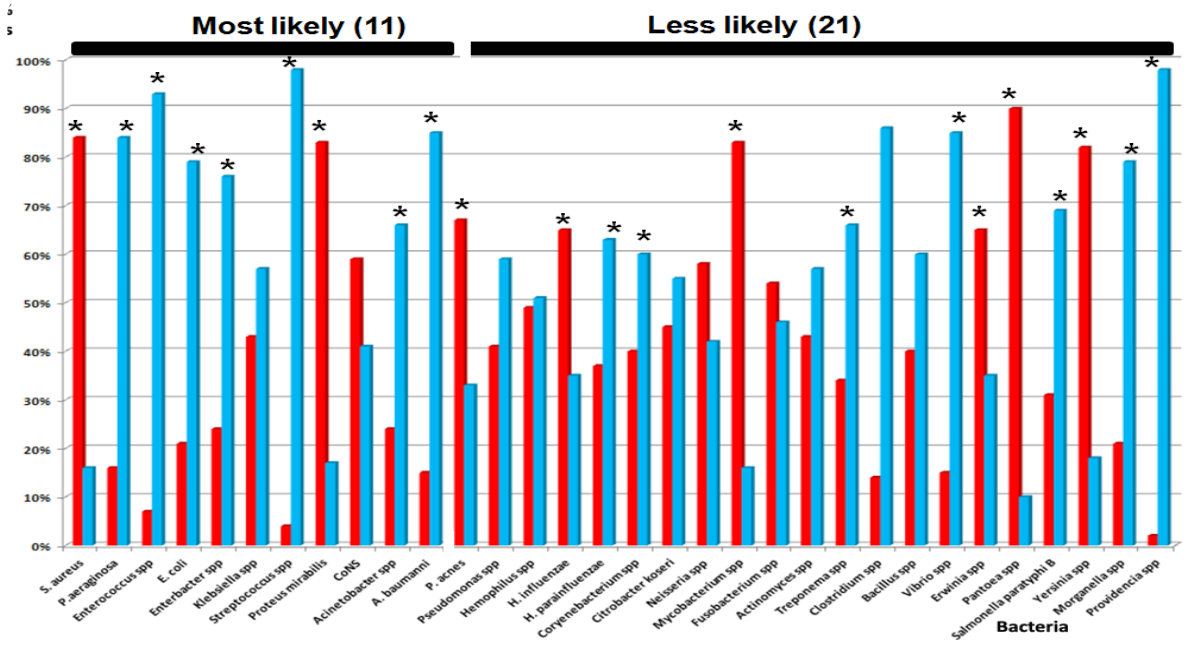

Figure 4: Relative abundances of bacterial genera/species (n=32) detected in CL (red bars) and non-CL ulcers (blue bars). Asterisks designate statistical significance ($P < 0.05$). *Klebsiella* spp included *K. pneumoniae*, *Acinetobacter* spp included all species except *A. baumannii, Pseudomonas* spp included all species except *P. aeruginosa*, *Propionibacterim* spp included *P. avidum* (See supplementary files S1).

former is known to be pathogenic and not skin flora, while the latter is usually skin flora which can become pathogenic due to reasons like weakened immune system and mode of entry like bite (Chiller, Selkin et al., 2001; Moet, Jones et al., 2007). The relative abundances of the two groups were compared in CL and non-CL ulcers as shown in Figure 4. In the first group, the relative abundance of *E coli* (79%), *Pseudomonas aeruginosa* (84%), *Enterobacter* (76%), *Enterococcus* (93%) and *Acinetobacter* species (66%) were significantly higher in non-CL ulcers (*P<0.05*), while *Staphylococcus aureus* and Proteus mirabilis were higher in CL ulcers which represent (84%) and (83%) of reads, respectively (P<0.05). CoNS (Coagulase-negative *Staphylococci*) and Klebsiella spp. were comparable in CL and non-CL (P>0.05). The variation in the relative abundances of OTUs in CL and non-CL microbiome as a function of *Leishmania* species, prior treatment, age and gender are illustrated in the heat map (Figure 5). The bacterial genera/ species across the patients' samples clustered intermediately together on three occasions (2-6 on intensity color scale shown in Figure. 5). In the first cluster, *Corynebacterium* spp and *Pseudomonas* spp grouped with samples of CL ulcers. The second cluster showed *Treponema* spp, *Haemophilus* spp, *Neisseria* spp, and *Actinomyces* spp grouping mainly in samples of non-CL ulcers. While in the third cluster, *P. aeruginosa, E. coli*, *A. baumannii* and other *Acinetobacter* spp clustered

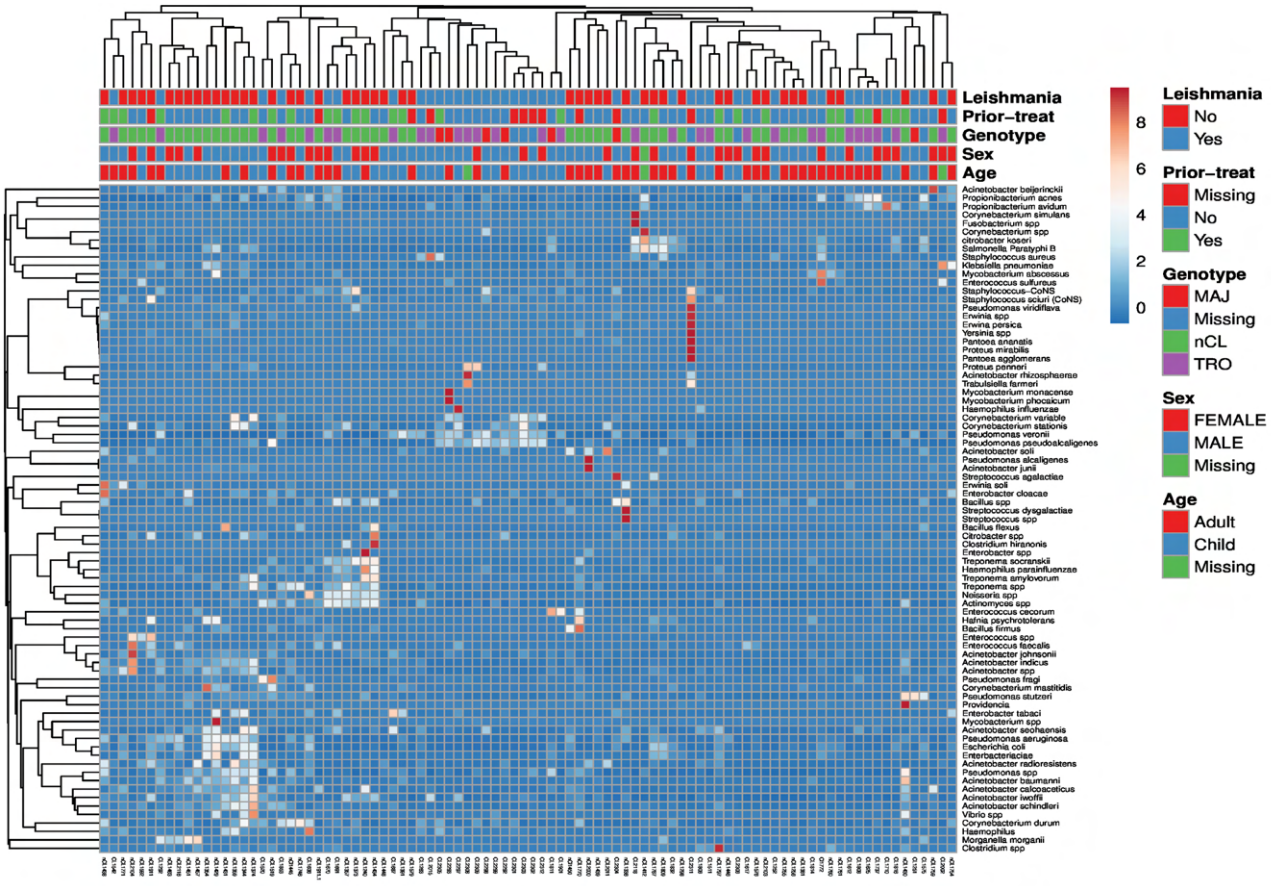

Figure 5. Heatmap of the number of reads of the bacterial genera/species (rows) and samples of patients with CL and non-CL ulcers (columns). Annotations on top of the heatmap show clustering of the samples based on demography and clinical picture parameters. Dendrograms are shown on the top and left side. The heat map has been color-scaled (79 rows) and clustered (both 92 columns and 79 rows). The legend of abundances (top right) is colored from low abundance (blue) to high abundance (red).

in non-CL ulcers. One sample (CL2311) had a strong relative abundance of *Enterobacteriaceae* families such as *Pantoea* spp, *Proteus* spp, *Erwinia* spp and *Yersinia* spp (Figure. 5). No significant correlation was found between treatment before sampling and those without prior-treatment (Figure. 5).

Discussion

It is well-known that secondary bacterial infections can prolong the disease duration, increase tissue necrosis and result in the formation of scar (Layegh, Ghazvini et al., 2015). A study conducted on Balb/c mice reported that CL lesions experimentally produced by *L. major* may facilitate concomitant bacterial infections that interfere with the healing process. According to the results of this work, following treatment, the rate of disappearance of bacteria inoculated into the base of the tail of uninfected Balb/c mice was much faster than that of bacteria inoculated into experimentally induced CL lesions. These results suggested that *Leishmania* parasites and their metabolites might induce local immunosuppression in the lesion and thus facilitate bacterial infections (el-On, Sneier et al., 1992; Isaac-Marquez and Lezama-Davila, 2003). Our results showed that the detection rate of bacterial DNA-using 16s rRNA-PCR- in CL lesions was 28.7%.

In both groups (CL and non-CL ulcers), most of the sequences were assigned to five core phyla: *Proteobacteria, Actinobacteria, Firmicutes, Fusobacteria and Spirochaetes*; which are described as the most abundant phyla of the healthy skin microbiome(Grice, Kong et al., 2009). At the phylum level, a similar microbial community with significantly different proportions was found in the microbiome of localized lesions taken from CL patients in Brazil: Firmicutes (54.3%), *Actinobacteria* (11.7%), *Fusobacteria* (11.6%), *Proteobacteria* (8.7%), and *Bacteroidetes* (5.1%) (Salgado, Queiroz et al., 2016). In this study, the most abundant OTUs in CL group were from *Actinobacteria* phylum (52%) followed by *Proteobacteria* (33%) which may indicate a specific microbiome for Palestinian CL patients. This unique difference between populations is supported partly by the findings of the Human Microbiome Project-HMP (http://hmpdacc.org) that showed intra and inter-individual variation in the microbiome composition within the same nation due to genetic, environmental, dietary and other unknown reasons (Human Microbiome Project, 2012). In addition, age, site of lesion and microbial interaction play role composition of skin microbiota (Mukherjee, Mitra et al., 2016; Oh, Conlan et al., 2012). Bacterial diversity was lower in CL lesions compared to those of non-CL which was associated with opportunistic contamination by commensal bacteria. *E. coli* and *P. aeruginosa*, dominated the scene in non-CL compared to CL lesions. Strains of *E. coli* were frequently isolated from skin and soft tissue infections and exhibited a remarkable virulence comparable to *E. coli* strains isolated from urinary tract infections and bacteremia (Petkovsek, Elersic et al., 2009). Moreover, *P. aeruginosa* a multidrug resistant pathogen also can cause diverse infections such as burn injuries and otitis externa (Hirsch and Tam, 2010). However, microbiome studies can not always identify the actual causative agent of infection by comparing the relative abundances and prevalence of pathogens, since low abundances of known causative pathogens were documented in skin ulcers (Van Leuvenhaege, Vandelannoote et al., 2017). In fact, several studies on skin microbiota have shown that most areas of the skin surface are too dry habitat for multiplication of gram-negative bacilli (Fontes, Carvalho et al., 2005). Nonetheless, *P. aeruginosa*, has been isolated from clinically-infected lesions (Moet, Jones et al., 2007). It has been demonstrated that Gram-negative bacteria, particularly *Proteobacteria*, represent an important component of the skin microbiota and not environmental contaminants or microbiota from other body sites (Cosseau, Romano-Bertrand et al., 2016; Grice, Kong et al., 2008).

On the other hand, the association of known Gram-positive species belonging to *Firmicutes* and *Actinobacteria* phyla to skin lesions has been previously established (Dodson, Craig et al., 2010; Kuehnert, Kruszon-Moran et al., 2006).

Our results revealed that 84% of S. aureus were in CL ulcers, which are colonizer bacteria in one third of the normal population, however, putting them into the risk of infections. The *S. aureus* sequences were relatively low which could be due to microbial interaction with CoNS such as *S. epidermidis*, which inhibit *S. aureus* in a phenomenon called colonization resistant (Buffie and Pamer, 2013). We concluded that presence of pathogenic bacteria, in CL ulcers, is strongly suggestive of a concomitant bacterial infection which is in line with culture-dependent studies from Iraq, Iran, Sudan, Latin America and Brazil that showed *S. aureus* is prevalent in CL lesions (AlSamarai and AlObaidi, 2009; Fontes, Carvalho et al., 2005; Sadeghian, Ziaei et al., 2011; Ziaie and Sadeghian, 2008). The presence of pathobiome directly affects the treatment of CL lesions by delay in healing, disfiguring of infection site, and scar formation, therefore, several studies indicated the need to eliminate bacterial purulent infections by antibiotic treatment before antimonial administration to CL patients (Edrissian, Mohammadi et al., 1990; Isaac-Marquez and Lezama-Davila, 2003; Van Der Vliet, Le Guern et al., 2006). *Proteus mirabilis*- 59% in CL ulcers in this study-has also been isolated from abscesses and burns (Mistry, Scott et al., 2010). Therefore, our results suggest the need to consider bacterial infections in the treatment regimen of patients with CL. We found highly abundant 16S rRNA sequences that matched *P. acnes*, of which 67% were captured in CL ulcers (Fig 3). This bacterial species is part of the normal microbiota of the skin, which thrives mainly on sebaceous body sites such as a face that formed approximately 40% of infected sites (Mukherjee, Mitra et al., 2016; Oh, Conlan et al., 2012). This high percentage of *P. acnes* induces the growth of *S. aureus* in and around the ulcer (Wollenberg, Claesen et al., 2014).

A study conducted on mice in 2017 showed that the use of topical antibiotics change the microbial make up of skin long after application. Compared to untreated mice, those who were treated with triple antibiotic ointment (TAO) exhibited an immediate and significant decrease in bacterial diversity starting after the first day of treatment and maintained for greater than 1 week post-treatment (SanMiguel, Meisel et al., 2017). In Palestine, particularly in rural areas, it is a common practice that some patients undertake empirical remedies and receive antibiotic drugs indiscriminately. Such practice may lead to changes in skin microbiota termed dysbiosis where beneficial bacteria can become pathogenic (Iebba, Totino et al., 2016). We predicted that prior usage of topical or systemic medications might influence the skin microbiome. Therefore, to investigate this possible relationship, the study population was grouped into patients who reported no use of medications and those who reported use of topical and/or oral antibiotics before sampling. Although 29% of the tested lesions were treated before sampling, we found no significant differences between the microbiome of lesions in both CL and non-CL groups and prior antibiotic use.

This study was restricted to samples that were collected from CL-suspected patients who were referred to the Ministry of Health clinics for laboratory diagnosis. Therefore, our study limitations included: First, no samples were taken from healthy people living in the same area from which the patient samples were collected, in order to compare the results with them as control group. Second, it was difficult to recruit CL and non-CL samples with the exact body site match as CL is not restricted to a specific body site. However, several studies showed some inter- individual differences in the skin microbiome even when matched for body site whereas other studies described the differences in the skin microbiome using samples from patients and control subjects from unmatched body sites (Fahlen, Engstrand et al., 2012).

In conclusion, the CL and non-CL ulcers have no significant difference in their microbial communities, yet, non-CL was more scattered. Nonetheless, this retrospective study does highlight that CL lesions are colonized with pathogenic bacteria that might complicate the clinical picture of CL and subsequently cause

difficulty in diagnosis and delay in treatment. The most abundant phyla accounted for the majority of reads in the samples were members of the universal core of skin microbiome. A prospective study is needed to investigate the effect of these specific bacteria on healing time and to monitor the microbiome changes during the ulcer-healing course.

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Assessment of job satisfaction and job related stress among pharmacists in the West Bank, Palestine

RESEARCH

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A B S T R A C T

Job satisfaction is considered one of the essential factors contributing to a person's motivation, productivity and overall well-being, the present study aimes to assess job satisfaction and job-related stress levels among pharmacists that are currently registered and practicing in Palestine. we report a cross-sectional survey, including measures of satisfaction and stress (Health Professions Stress Inventory) questionnaire. Data were analysed using descriptive statistics, t-tests and one way ANOVAs. The significance level was set at *P<0.05*. Out of 694 questionnaires distributed, 576 were returned; 14 were not completed and excluded from analysis giving a net of 554 (79.8%) participants. Most of the respondents in the analysis sample were female (58.3%) working in community pharmacies (73.6%). The level of job satisfaction was 58.5%, the variables that contributed to the statistically significant, differences in the degree of job satisfaction were the region (*p<0.001*) and the monthly income (*p<0.001*). The t-tests and ANOVA analyses revealed that hospital pharmacists were the least likely to respond that job conflicts with family responsibility as a source of stress compared with community pharmacists (3.11 vs 2.14; *p<0.001*) and least likely scores in the professional recognition domain (3.21 vs. 2.79; *p =0.04*), respectively. Other job stressors like excessive work load, lack of promotion opportunities and poor physician pharmacists' relationship have also been reported. Work life of pharmacists should be enhanced in order to improve their motivation and ability, because failure to reduce stress among workers puts both pharmacists and patients at risk.

Keywords: Community pharmacists, Hospital pharmacists, Job Satisfaction. Job related-stress, Palestine.

Introduction

After a job evaluation, one can experience a pleasing emotional response, which is known as job satisfaction, which is reflected in a person's attitude toward their job(Baloch 2009).Job satisfaction is considered one of the essential factors contributing to a person's motivation, productivity and overall well-being (Sansgiry and Ngo 2003). Evaluation of Pharmacist job satisfaction is of vital importance due to the fact that it reflects stability in the work place since pharmacist commitment to workplace, turnover or consideration to leave the profession are all related to job satisfaction (Desselle and Peirce 2011, Ferguson, Ashcroft et al. 2011). Furthermore, poor job satisfaction of pharmacist may affect job performance, patient care or patient interaction (Saari and Judge 2004, Kreling, Doucette et al. 2006), worst of all it can lead to dispensing errors

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and increased risk of patient harm(Mott, Doucette et al. 2004, Faragher, Cass et al. 2013, Mukattash, Alzoubi et al. 2016). Another factor that may affect pharmacist performance is stress. The job of pharmacist requires a a great deal of responsibility and a heavy workload. Thus, as with numerous health professions, there is an increasing stress level in the pharmacy (Felton 1998, Merrick 2000, Edwards and Burnard 2003, Keil 2004, McNeely 2005, Stafford-Brown and Pakenham 2012). The elevated stress that a pharmacist experiences may be attributed to unreasonable workload expectations, long work hours and poor communication. Previous research suggests that one of the main indicators of workplace stress is interpersonal conflict with co-workers or physicians. In some instances, this may be a reason why pharmacists choose to leave the profession (Herzog 2000, Kälvemark, Höglund et al. 2004, Austin, Gregory et al. 2010). Part of a pharmacist's stress is due to the fact that establishing a relationship of trust and collaboration with physicians is often a challenge(Smith, Ray et al. 2002, Snyder, Zillich et al. 2010). In Palestine, job satisfaction and job stress in the pharmacy profession has not been studied. To date, this study represents the first time that job satisfaction and job-related stress levels have been assessed among pharmacists that are currently registered and practicing in Palestine.

Methods

Study setting and design

The study was conducted in the West Bank of Palestine between April and October in 2018 and was cross-sectional and questionnaire based. The West Bank is separated into three regions, including the Northern region, the Central region and the Southern region.

Study participants

The finally target population for this study included all registered Palestinian Pharmaceutical Association (PPA) member pharmacists that were working in different pharmaceutical sectors (community pharmacy,private pharmacy, hospital pharmacy, pharmaceutical company and medical representative) in the West Bank. At the time of the study, there were 4100 PPA registered pharmacists in the West Bank.

Study Sample

A proportionate stratified random sampling technique was used to achieve the study aims and goals. In this sampling technique, the sample size selected from each stratum is proportionate to the relative size of that stratum in the study population. The sample size was calculated using the Raosoft® sample size calculator. The sample size was 256 pharmacists, which was the minimum recommended number.

Ethical approval

The Al-Quds University Research Ethics Committee (REC) approved the protocol for this study; Ref No. (39/REC/2018). An explanation of the study rationale was provided and pharmacists were confirmed that the survey would measure job satisfaction, their attitudes and stress. Willingness to participate was a fundamental condition in the study.

Study instrument

The questionnaire was developed by evaluating the literature and the questionnaires that were utilized in similar studies (McCann, Hughes et al. 2009). The questionnaire consisted of 4 sections. The first section was the socio-demographic data (e.g. age and gender). The second section included four points related to job satisfaction. The third section included a subsection of questions (30 items) that were from the Health Professions Stress Inventory (HPSI), which was established by Wolfgang (Wolfgang 1988). The HPSI questionnaire consists of 4 areas, including patient care responsibility (7 items), job conflicts (8 items), professional recognition (8 items) and professional uncertainty (7 items). For this part, the pharmacists were asked to rate 30 situations using a five-point Likert scale (the responses ranged from 1= never stressed to 5 =frequently stressed). The fourth section was optional and was for free text responses

on other characteristics of job satisfaction and stress, such as physicians' cooperation, sick leave and overtime work. The questionnaire was developed in English language and translated to Arabic by a committee of three Professors, two of them in Pharmacy domain. A statistics professor first performed the content validity, and then, the questionnaire was translated back into English to ensure accuracy of the tool. A pilot questionnaire was first used using 30 pharmacists in order to assess the research tools, with respect to acceptability, applicability, and the period. The stability of the total degree of the stability coefficient was calculated to verify the stability of the tool for the fields of study using the Cronbach Alpha stability equation and the total score (0.896). This resulting value indicated that the tool was consistent with the goals of the study.

Data Analysis

All the statistical analyses were done using SPSS version 21 (SPSS Inc., Illinois, USA), and *P<0.05* was considered significant. The statistical processing of the data was performed by extracting the mean and standard deviation of each paragraph of the questionnaire. A t-test and a one-way ANOVA were performed, and the Pearson correlation coefficient was calculated. A least significant differences (LSD) post-hoc test was used to identify the differences between the groups when significant differences were found and when the independent variable was composed of more than one level. The degree of average responses on a four-point scale of ≤ 2.00 was considered low, 2.01 – 3.00 was considered medium and ≥ 3.01 was considered high. The degree of average responses on a five-point scale of ≤ 2.33 was considered low, 2.34 – 3.67 was considered medium and ≥ 3.68 was considered high.

Results

Of the 694 questionnaires distributed, 576 were returned; 14 were not completed and excluded from analysis giving a net of 554 (79.8%) participants. Table 1 is a summary of the socio-demographic characteristics of the respondents. Most of the respondents in the analysis sample

Table 1: Participant pharmacists demographic data (N =554)

Variable		N	%
Region	North	281	50.7
	South	115	20.8
	Middle	158	28.5
Gender	Male	231	41.7
	Female	323	58.3
Age	From 22-30 years	321	57.9
	From 31-40 tears	137	24.7
	More than 40 years	96	17.3
Marital Status	Single	186	33.6
	Married	362	65.3
	Widowed /Divorced	6	1.1
Graduation year	1990 and before	31	5.6
	1991-2000	84	15.2
	2001-2010	149	26.9
	2011 and above	290	52.3
Pharmacy License	1990 and before	27	4.9
	1991-2000	80	14.4
	2001-2010	144	26.0
	2011 and above	303	54.7
Daily Work	Less than 6 hours	26	4.7
	6-8	373	67.3
	9-12	121	21.8
	More than 12	34	6.1
Academic Degree	B. Sc.	453	81.8
	Pharm. D	61	11.0
	Post Bach. Pharm. D	40	7.2
Practical Setting	Community Pharmacy	408	73.6
	Hospital	79	14.3
	Pharmaceutical Company	37	6.7
	Medical Representative	30	5.4
Position	Owner of the pharmacy	172	29.8
	employee	382	67.1
Experience	< a year	114	20.6
	1 - 3	165	29.8
	4 - 6	91	16.4
	7 - 10	51	9.2
	> 10 years	133	24.0
Monthly income	< 1000 NIS	9	1.6
	1000-2000	171	30.9
	2001-3000	158	28.5
	3001-4000	87	15.7
	49001-5000	51	9.2
	5001-6000	36	6.5
	> 6000 NIS	42	7.6
Job Status	Full time job	372	67.1
	Part time job	182	32.9

were females (58.3%) working in community pharmacies (73.6%), the greatest proportion of the respondents lived in the North (50.7%). The young pharmacists (22-30 years) who graduated and obtained pharmacy license after 2011 were more than (50%) of the sample population. According to last the two columns in Table 2, among their daily duties, pharmacists devoted 55% of their patient-associated activities to medication dispensing, 35% of their time was for patients consultation and drug use management, while 29% of their time was dedicated to pharmaceutical care, 28% to education, and 24% was for business management and marketing. Amazingly, only 16% of the time was dedicated to clinical pharmacist services, and 15% was for disease management. When pharmacists were asked about any plans for leaving the current employment next year; 30% of the respondents intended to leave work next year, and 34.3% of them had a possibility to leave their work. The reason for leaving work was 22.2% due to salary, 21.3% due to workload and work schedule.

Job satisfaction

The level of job satisfaction is presented in Table 3, showing that 58.5% were satisfied and 21.7% were dissatisfied. A total of 54.6% of the respondents reported satisfaction with their current job, and 14% were dissatisfied. As part of the questionnaire, the pharmacists were asked about whether or not they would select pharmacy as a career again if they had a choice, and 33.4% of the respondents said that they would select the specialty of pharmacy again, while 23% of them responded that when they leave work, they have a ''bad'' feeling, because they do not enjoy their job. Job satisfaction and work pressure showed a statistically significant inverse relationship (Pearson $r=-0.112$, $p < 0.01$). The variables that contributed to the statistically significant differences in the degree of job satisfaction were

Table 3. Numbers and percentages of the study sample responses at the Level of Job Satisfaction

Item	N	%
Are you satisfied with your job?		
Very Dissatisfied	51	9.2
Dissatisfied	69	12.5
Neither	110	19.9
Satisfied	200	36.1
Very satisfied	124	22.4
Are you satisfied with Your present job compared to others		
Very Dissatisfied	33	6.0
Dissatisfied	44	7.9
Neither	119	21.5
Satisfied	208	37.5
Very satisfied	150	27.1
How often do you leave work with a ''bad'' feeling, a feeling that you are doing something you do not enjoy		
Never	69	12.5
Rarely	215	38.8
Sometimes	145	26.2
Often (most of the time)	118	21.3
Very Often (all of the time)	7	1.3
If you could start your career over, would you choose to do pharmacy again?		
Definitely No	174	31.4
Don't know	88	15.9
May be	107	19.3
Definite yes	185	33.4

Table 2. Pharmacists' work activities and work environment (n %)

	No of responses	< 20%	21-40%	41-60%	61-80%	81-100%
Medication Dispensing	40 (7.2)	48 (8.7)	53 9.6)	109 (19.7)	105(19.0)	199 (35.9)
Consultation	41 (7.4)	177 (31.9)	88 (15.9)	55 (9.9)	98 (17.7)	95 (17.1)
Drug Use Management	75 (13.5)	177 (31.9)	45 (8.1)	58 (10.5)	76 (13.7)	122 (22.0)
Pharmaceutical care	118 (21.3)	164 (29.6)	56 (10.1)	54 (9.7)	86 (15.5)	76 (13.7)
Clinical pharmacist services	261 (47.1)	106 (19.1)	33 (6.0)	68 (12.3)	34 (6.1)	52 (9.4)
Disease management	244 (44.0)	125 (22.6)	47 (8.5)	57 (10.3)	53 (9.6)	28 (5.1)
Business Management	182 (32.9)	136 (24.5)	52 (9.4)	54 (9.7)	6.1 (11.0)	69 (12.5)
Marketing	176 (31.8)	117 (21.1)	55 (9.9)	74 (13.4)	69 (12.5)	63 (11.4)
Education	163 (29.4)	126 (22.7)	48 (8.7)	58 (10.5)	82 (14.8)	77 (13.9)

the region (*p<0.001*) and the monthly income (*p<0.001*). Thus, the LSD results for the region variable were examined to show the direction of the differences, showing that the differences were in favor of the middle region and for incomes greater than 6,000 NIS. However, for the domain of leaving work with a good feeling, the results show that for gender the differences is in favor of females (*p<0.001*), for practical settings variable, pharmacy hospital was favored *(p<0.001*) and for position variable in favor of pharmacy owner (*p<0.001*).

Job stress

Table 4 represents the stress domains, which consist of 30 items.

Table 4. The means and standard deviations of scores for domains of health professions stress inventory (HPSI)

#	Domain	No. of Items	Possible range of scores	M	SD
1	Patient care	8	1-5	3.13	0.650
2	Professional recognition	7	1-5	2.77	0.623
3	Job conflicts	7	1-5	2.47	0.606
4	Professional uncertainty	8	1-5	2.31	0.640
	Total	30	1-5	2.61	0.485

The responses ranged from 1, which was for never stressed, to 5, which was for frequently stressed. The overall mean of the stress scores was 2.61 and the standard deviation was 0.485, which indicated that the level of work stress of the pharmacists was medium. Patient Care has the highest average score (3.13 ±0.65) and the lowest was the Professional uncertainty score (2.31 ±0.48). The four job situations that the pharmacists perceived as the most stressful were as follows: "Trying to meet society's expectations for high-quality medical care" in the patient care domain(3.54± 1.01); "Having job duties which conflict with family responsibilities" in the job conflict domain (2.91± 1.1); "Possessing inadequate information regarding a patient's medical condition" in the profession uncertainty domain (2.57± 1.04); and "Feeling that opportunities for advancement on the job are poor" in the profession recognition domain (3.3± 1.1). Community pharmacists feel stress regarding responsibility for patient outcomes and phone call interruptions, while hospital pharmacists feeling stress when they debate with supervisors and or administrators when they have too much work to do.

Table 5 shows the t-tests and ANOVA analyses revealed that hospital pharmacists were the least likely to respond that job conflicts with family responsibility as a source of stress compared with community pharmacists (3.11 vs 2.14; *p<0.001*) and least likely scores in the professional recognition domain (3.21 vs. 2.79; *p =0.04*), respectively. A higher mean stress score was reported by newly practicing (i.e. less than 3 years) community pharmacists compared with pharmacists who were practicing > 3 years, with regard to the domains of patient care responsibility (3.41 vs. 2.11, *p <0.001*) and professional uncertainty (2.78 vs. 2.14, *p = 0.005*). The top situation that new pharmacists considered to be the most stressful was "Fearing that a mistake will be made in the treatment of a patient."

Physician Cooperation

Thirty five percent (35%) of the respondents to this question indicated that there is a very good professional relationship between the doctor and pharmacist, on the other hand 45% of the respondents indicated that physicians cooperation with pharmacist is not enough or rare. In general, some physicians are highly cooperative while others cling on to their opinion even if they are wrong.

Sick leave

It is a difficult situation when pharmacists ask for casual or sick leaves, because it is a struggle to find a replacement, and 30% of the respondents answered that there was no available paid sick leave for them, 40% of the respondents indicated that it was not enough for them, and the rest said it was there when necessary.

Table 5. The means, standard deviations, and level of significant of the responses of the study sample members to level of work stress of participant pharmacists

		Patient care			Professional recognition			Job conflicts			Professional uncertainty			Total		
Variable	N	M	SD	Sig.	M	SD	Sig.	M	SD	Sig.	M	SD	Sig.	M	SD	Sig.
Region																
North	281	2.57	0.647	0.812	3.11	0.662	0.128	2.46	0.635	0.407	2.32	0.649	0.481	2.60	0.511	0.427
South	115	2.57	0.584		3.24	0.638		2.54	0.545		2.35	0.596		2.66	0.435	
Middle	158	2.60	0.609		3.10	0.633		2.45	0.595		2.26	0.654		2.59	0.473	
Gender																
Male	231	2.55	0.615	0.332	3.13	0.663	0.921	2.51	0.597	0.189	2.27	0.661	0.319	2.61	0.480	0.892
Female	323	2.60	0.628		3.14	0.643		2.44	0.612		2.33	0.624		2.62	0.489	
Academic Degree																
B. Sc.	453	2.55	0.617	0.054	3.11	0.662	0.176	2.47	0.606	0.015*	2.29	0.632	0.363	2.60	0.488	0.043*
Pharm. D Post Bach.	61	2.62	0.607		3.20	0.499		2.37	0.558		2.38	0.658		2.62	0.411	
Pharm. D	40	2.79	0.684		3.30	0.707		2.72	0.632		2.40	0.697		2.80	0.524	
Practical Setting Community																
Pharmacy	408	2.62	0.603	0.251	3.21	0.592	0.046*	3.11	0.571	0.001*	2.35	0.624	0.125	2.65	0.436	0.087
Hospital Pharmaceutical	79	2.57	0.668		2.79	0.748		2.14	0.639		2.18	0.587		2.54	0.538	
Company Medical	37	2.65	0.465		2.90	0.744		2.71	0.560		2.29	0.605		2.64	0.475	
Representative	30	2.85	0.913		3.07	0.440		2.98	0.570		2.67	0.669		2.90	0.500	
Position																
Owner of the pharmacy	172	2.51	0.556	0.246	3.17	0.570	0.170	2.44	0.570	0.154	2.25	0.605	0.359	2.58	0.433	0.622
employee	382	2.60	0.646		3.13	0.679		2.48	0.609		2.33	0.655		2.62	0.500	
Experience																
< a year	114	3.41	0.611	0.001*	3.27	0.631	0.058*	2.47	0.669	0.324	2.78	0.630	0.005*	2.69	0.515	0.008*
1 - 3	165	3.12	0.643		3.16	0.550		2.50	0.554		2.35	0.636		2.65	0.424	
4 - 6	91	2.63	0.628		3.01	0.723		2.51	0.586		2.31	0.655		2.61	0.518	
7 - 10	51	2.11	0.500		3.15	0.565		2.56	0.580		2.27	0.560		2.65	0.383	
> 10 years	133	2.38	0.613		3.06	0.738		2.38	0.633		2.14	0.644		2.49	0.521	
Monthly income																
< 1000 NIS	9	2.65	0.328	0.066	3.52	0.446	0.388	2.52	0.458	0.456	2.43	0.343	0.056	2.76	0.249	0.275
1000-2000	171	2.64	0.675		3.17	0.645		2.43	0.623		2.36	0.648		2.64	0.519	
2001-3000	158	2.62	0.624		3.11	0.681		2.47	0.624		2.33	0.685		2.62	0.516	
3001-4000	87	2.50	0.627		3.05	0.705		2.58	0.608		2.25	0.641		2.59	0.475	
49001-5000	51	2.34	0.550		3.11	0.614		2.39	0.579		2.04	0.549		2.47	0.404	
5001-6000	36	2.57	0.555		3.09	0.485		2.43	0.591		2.25	0.519		2.57	0.413	
> 6000 NIS	42	2.59	0.515		3.23	0.630		2.57	0.527		2.42	0.609		2.69	0.410	
Job Status																
Full time job	357	2.54	0.601	0.036*	3.10	0.649	0.125	2.50	0.598	0.359	2.30	0.633	0.752	2.60	0.469	0.388
Part time job	167	2.68	0.624		3.22	0.626		2.44	0.608		2.33	0.636		2.65	0.490	

Paid overtime

Inadequate pay and few opportunities for job advancement were often being one of the stress sources among pharmacist. Most of the answers concentrated that sometimes there are extra working hours but without pay, or do not count any extra hour as overtime.

Discussion

Until now, the degree of stress and job satisfaction among Palestinian pharmacists has not been studied. Generally, results indicate medium scores of job satisfaction among Palestinian pharmacists; most are satisfied with being pharmacist and are satisfied with their present job compared to others. The pharmacists questioned in the present study presented a greater degree of job satisfaction in comparison to other studies. For example, 57% of community pharmacists in the UK were reported to be content with their current job "most of the time" (McCann, Hughes et al. 2009), and in other study, 38% of Jordanian pharmacists reported that they were satisfied with their job (Al Khalidi and Wazaify 2013). Moderate levels of job dissatisfaction were found for community and clinical pharmacists, while clinical pharmacists reported higher level of stress than community pharmacists. These findings are consistent with previous research (Maio, Goldfarb et al. 2004, Hassell 2006, Liu and White 2011, Munger, Gordon et al. 2013, Johnson, O'connor et al. 2014). Although dispensing is an essential component in the delivery of any pharmaceutical service, community pharmacists often feel unappreciated and get a sense that their abilities are not being employed in the patient care process. For example, a study in Arizona revealed that job satisfaction was significantly correlated with the apparent application of their skills amid community and hospital pharmacists(Cox and Fitzpatrick 1999). Our study also revealed that numerous controlling factors played a statistically significant role in influencing a pharmacist's job satisfaction and intentions to leave their job; these included the pharmacist's education level, gender, and monthly income. For example, male pharmacists were more likely to have intentions to leave their job compared to female pharmacists. The reason for this may be that female pharmacists are typically more stable in their respective position, while male pharmacists are more willing to seek out higher paying jobs in other pharmaceutical sectors, such as pharmaceutical sales(Lin, Yeh et al. 2007, Seston, Hassell et al. 2009, Majd, Hashemian et al. 2012). The findings showed that job satisfaction was not statistically different among Palestinian pharmacists based on whether the pharmacists owned the pharmacy or not. The only exception was regarding the question of "leaving work with a ''bad'' feeling", and the results indicated a higher job satisfaction among the owners. This could be explained by the fact that salary increases with pharmacy ownership; this may explain the higher job satisfaction for the pharmacy owner and pharmacists with high monthly wage of more than 6000NIS(Salameh and Hamdan 2007). Job satisfaction among Palestinian pharmacists due to the years of experience was not significantly different. Our results contradict other studies, which indicate that pharmacists with more years of experience have a greater sense of job satisfaction.

With regard to stress, in the present study, the level of perceived stress was higher (an overall mean of 2.61) than the overall mean stress reported in Northern Ireland study (McCann, Hughes et al. 2009). Furthermore, the level reported in our study was also greater than the Swedish normative data, which derived from 3406 individuals, showing a mean perceived stress scale score of 13.96 (Nordin and Nordin 2013). "Feeling that opportunities for advancement on the job are poor" was a major cause of the stress reported by pharmacists as was not obtaining prospects for improvement. In addition, "Not having opportunities to share feelings and experiences with colleagues" and "Not being able to use your abilities to the fullest extent on the job" were important concerns for a

great number of pharmacists. On the other hand, as mentioned by Doody (Doody 2012), "Not being recognized as a true health professional by other health professionals" was one of the main causes of stress for pharmacists, in addition to not being acknowledged by the general public. The most common cause of stress among the respondents was "possessing inadequate information regarding a patient's medical condition."

It is possible that this was related to the high level of accountability that they have. On the other hand, as mentioned in previous publications (Doody 2012), the most common cause of stress is reported to be related to "having to balance new roles with existing responsibilities." The reason for this stress area, might be due to increased pharmacy regulations, which equates to an increased level of responsibility. Moreover, community pharmacists' stress may be related to the fact that pharmacist play a dual role, on the one hand there is a business component to the profession in addition to being a health care professional(Bond, Matheson et al. 2000, Resnik, Ranelli et al. 2000, Austin, Gregory et al. 2010). There is a common expectation for community pharmacists to provide patient pharmaceutical care in spite of inadequate information available about patient condition. This stressful situation causes the pharmacist to feel uncertain of how to counsel the patient (Resnik, Ranelli et al. 2000). The main cause of stress for clinical or hospital pharmacists appears to be due to daily job conflicts with other health care professionals. Participation of clinical pharmacists in Palestinian hospitals is relatively new (Mukattash, Hayajneh et al. 2016, Khdour, Hallak et al. 2018), as such they may not be accepted by physicians and other health care professionals that may not acknowledge the newer clinical services delivered through the pharmacist (Tahaineh, Wazaify et al. 2009). Similarly, a study in Riyadh found job-related stress factors, such as the setting of the pharmacy and years of experience, had a significant effect on the stress level related to the responsibility of patient care (Slimane 2017). A possible explanation for this is that pharmacists, whether they work in a chain pharmacy or work an independent community pharmacy, feel that their professional training is not utilized as much as their fellow health care professionals, and this would include patient-oriented functions, such as clinical-related activities and direct patient care. Generally, dispensing pharmacists might not feel appreciated, such that the skills they obtained in their training are not being fully utilized for patient care. Based on the free text analysis, in which the respondents were permitting to express any other issues or concern, we found that for the community pharmacists, it was difficult to establish a relationship with the physicians, and 45% of the respondents responded that the physicians rarely cooperated with the and that whatever cooperation they received was not enough. Any cooperation usually rely on the physician personality, some physicians are cooperative while other clings to their opinion even if they are wrong. For example, new doctors in all specialties are more likely to interact with pharmacist than older doctors, and some may ask pharmacists to recommend the best drug choice (Allenet and Barry 2003). On the other hand, many pharmacists described that physicians will regularly refuse their suggestions for replacing unavailable drugs brands with available substitutes. Another challenge that pharmacists face is that it is difficult to ask for time off using casual or sick leave, because it is not easy to find a replacement. As such, 30% of the respondents answered that there was no available paid sick leave for them, and 40% of the respondents complained that they do not get enough time to rest when necessary. The pharmacists also reported that the interaction between physicians and pharmaceutical companies, which may lead to inappropriate prescribing, was also a source of stress and dissatisfaction. This finding is consistent with reports demonstrating that when drug samples are available physicians were likely to be influenced to prescribe a drug that is different from their preferred drug of choice.

Thus, evidence-based guideline compliance may be compromised (Chew, O'Young et al. 2000).

Study Limitations

Satisfaction and stress are topics that remain controversial and still have potential for producing bias. Therefore, caution should be considered when interpreting the results of this study. Furthermore, there may have been errors by the pharmacists in recollecting some information. However, in an effort to minimize these errors, at any given opportunity, the researcher made sure that the participants were fully aware of the fact that they were able to reach out to the researcher by phone or email for any extra clarification. The personal social, emotional and financial status of the pharmacists may affect the results. However, we did not examine these factors in detail in this study.

Conclusion

This study revealed that the participating pharmacists experienced moderate levels of job-related stress and dissatisfaction. Community pharmacists described less satisfaction compared to pharmacists working in a hospital setting. The study results suggested that ample attention must be given to the demands placed on pharmacists on the job and their interactions with healthcare professionals in order to improve the work life quality of the pharmacist. Policy makers must pay special attention to raise the levels of job satisfaction for pharmacists in order to improve their motivation and ability, because failure to reduce stress among workers puts both pharmacists and patients at risk.

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Availability of data and materials

The raw dataset may be provided by the corresponding author upon reasonable request.

Authors' contributions

MK was conceived the idea for the study, led study design. JS collected the data, entered the data into SPSS, and conducted the data analysis. HH interpreted the data, and drafting of manuscript; All authors read and approved the final manuscript and agreed on its submission.

Ethics approval and consent to participate

The study was approved and authorized by the Institutional research ethics committee (REC) of Al-Quds University (39/REC/2018). Verbal consent was also obtained from the participant pharmacists prior to the commencement of the study.

Consent for publications

N/A

Competing interest

The authors declare that they have no competing interest.

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Links between nutrition, life style habits and academic achievement in Palestinian schoolchildren: A cross-sectional study

RESEARCH

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A B S T R A C T

Objective: To examine the association between nutrition, physical activity, lifestyle, the combined behavior effect, and the schoolchildren's academic achievement. *Design*: Observational and cross-sectional study. Setting: West Bank, Palestine. Participants: A group of schoolchildren (n=1945) in grades 5-9 (11-16 years). Measurements: Students were surveyed about the their 'dietary, physical activity (PA), leisure time activity, and academic achievement. Academic achievement was measured using students' marks in Arabic, English, math, science courses, and the total average score. The linear regression model was conducted to analyze the relationship between dietary, PA, combined behavior, and academic achievement, while adjusted for demographic confounders; body mass index (BMI), and parental education. *Results*: Findings indicated that healthy nutrition and adequate levels of PA significantly predict achievement scores. In both boys and girls, high academic achievement was associated with a high intake of fruits and vegetables (AOR: 1.1 (0.72-1.68); 1.18(0.81-1.7), and (AOR: 1.21(0.8-1.82); 1.33(0.93-1.91), respectively. In both girls and boys, high academic achievement was associated with low intake of soft drink, beverages (juice with sugar) and energy drink (AOR: (0.75(0.47-1.19), 0.85(0.58-1.27)); (0.99(0.63-1.57), 0.76(0.52-1.12)); (0.66(0.38-1.15), 0.49(0.27-0.89)), respectively. The active and healthy nutrition group scored higher on Arabic, English, math, science, and total average score. *Conclusions*: There is a strong relationship between healthy nutrition, acceptable PA, and the average academic achievement within schoolchildren. Findings emphasize the importance of linking nutrition, school PA, and health policies for improving cognitive functions and academic performance of Palestinian schoolchildren. Thus, school-based healthy lifestyle educational, health behaviors policy, and recommendation programs may have a greater effect on students' academic achievement.

Keywords: Nutrition, physical activity, academic achievement, schoolchildren, linear regression

Introduction

The effect of nutrition and health on academic performance has been approved by many research studies. Good health, nutrition, and physical activity improve cognitive functions among students and lead to better academic performance, particularly in school years it is linked to higher levels of future wealth and health (Pavleski, Koltovska-Nechoska, and Ivanjko 2017; Burkhalter and Hillman 2011; Janssen 2012; Forrest et al. 2013; Kristo et al. 2020). Various modifiable factors influence academic achievement, including socioeconomic status, nutrition,physical activity, and social wellbeing, which affect individual and community growth from birth through adolescence (Faught et al. 2017; Adolphus, Lawton, and Dye 2013; S. Y. Kim et

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al. 2016; Montazerifar, Karajibani, and Dashipour 2012; Stea and Torstveit 2014; Vadiveloo, Zhu, and Quatromoni 2009; Taras 2005; Dubuc, Aubertin-Leheudre, and Karelis 2020). Various studies have investigated the association between different nutritional aspects and academic achievement (Banerjee 2016; Marques et al. 2017; Bleiweiss-sande et al., 2019; Stea and Torstveit 2014). Healthy nutritional practices such as breakfast intake were investigated and found to be significantly associated with better academic attainment (Alhilabi and Payne 2018; Jeong 2019; Lundqvist and Vogel 2019). Moreover, diet quality has been linked to differences in academic achievement. Studies show that healthy food intake, including fruits, vegetables, grains, legumes and milk, and a lower intake of energy-rich, poor nutrient food groups (e.g. junk/fast food) are correlated with academic outcomes (Burrows et al. 2017; Asmare et al. 2018). Nutrient analysis including macro and micronutrients among different food groups was also studied to gain an in-depth analysis of adequacy and diversity of nutrient intake. For example, many studies report a positive effect of folate, iron, and B group vitamins, which may have different food sources, on academic achievement (Burrows et al. 2017). Physical activity is also an important determinant of educational attainment. Several studies demonstrate that higher marks are found in students who have regular physical activity, characterized by moderate- to vigorous-intensity physical activity daily (Castelli et al. 2015; Cook and Board 2013). Moreover, interventions to integrate physical education classes in schools have shown better academic outcomes (Castelli et al. 2015). Another important health indicator is obesity. It has been demonstrated in the literature that obesity, as an independent factor, correlates with poorer mental function and academic outcomes (Asigbee et al., 2018). The above associations were mostly studied individually. Few studies have investigated the interdependence between various variables and academic achievement (Hou et al. 2020; Florence et al., 2008; Castelli et al. 2015; Marques et al. 2017). In Palestine, there is a shortage in nationwide studies that examine the relationship between the above-mentioned variables and academic achievement. One study in the Gaza strip showed that the intake of fruit and vegetables was positively associated with school performance among adolescents in Gaza Strip while stunting was negatively associated with school performance (Abudayya et al. 2011). However, there is a need for an in-depth investigation of the dietary patterns prevailing in Palestine. Moreover, up to our knowledge, there's no study of the correlation between multiple health determinants and academic achievement. Therefore, this study aims to investigate the individual and combined behaviors effect of nutrition, physical activity, and obesity on academic achievement among primary and secondary school children in the West Bank, Palestine, in an attempt to make recommendations on the possible interventions using the above-mentioned factors, thereby enhancing academic performance.

Methods

Sample Population

Data were obtained from the national survey conducted in West Bank as a part of the Health Behavior in School-aged Children (HBSC) survey in 2013-2014. The study assessed the nutrition, physical and mental health of Palestinian schoolchildren in grades 5-9 (11-16 years).

A random sample of 2000 students were selected from the baseline database weighted for sex and grades. Out of 2000 students, 1945 students have accomplished the study criteria, the 55 students had missing data variables.

Measure

The survey used the modified Health Behavior in School-aged Children (HBSC) questionnaire (Roberts et al. 2007). The questionnaire contained questions about dietary intake, physical activity, leisure time activity, anthropometric measurements (height, weight, waist, and neck

circumstance), and academic achievement. Physical activity: Two scales were used to measure physical activity levels.The activity scale composed of three items: (1) For the last week, how many days were you physically active for more than 60 minutes, (2) number of hours playing sports outside school, (3) number of hours exercising per week. The second scale was used to measure the leisure time activity level. The scale items include (1) number of hours watching TV, (2) number of hours playing video games, and (3) number of hours using the internet. The 6 items asked for weekday and excluded the weekends. Based on the sum of the items, the respondents were analyzed using quartiles for both scales. The activity scale used the upper quartiles for identifying the physically active students, and the upper quartiles in the leisure time activity scale were used to identify the low and non-active students.

Diet: Dietary intake information was collected using face to face 24-hour food recall of one day intake and the food frequency questionnaire (FFQ). The food frequency scale was developed using the 8 food items scale. The food items were grouped into 8 categories based on similarity in nutrient profile (Frank et al. 1992).

These categories were: 1) vegetables; 2) fruits; 3) milk and other dairy products; 4) sweets and chocolate; 5) soft drinks; 7) beverages (juices and sugar); 8) energy drinks. Response categories were (1) never, (2) 1-2 times a week, (3) 3-4 times a week, and (4) 5-7 times a week (almost daily). Students were categorized into two consumption groups, healthy and unhealthy consumers. The scale sum was used to form the two groups. The healthy group include participants who were in the top 2 quartiles and had indicated that they did not eat any unhealthy items (soft drinks, chocolates, or energy drinks), and the unhealthy group include participants who had indicated they did not eat any of healthy nutrition items (vegetables, fruits, milk, and milk products) and were in the top 2 quartiles of the frequency of eating unhealthy foods. Academic achievement: The students' marks were obtained from the schools' marks records, the scores of all courses, and the total average score were obtained. In this study, we included the four major courses (Arabic Language, English language, math, and science) in addition to the total average score. Academic achievement was categorized into high and low scores. The grades higher than the mean were categorized as high, and grades lower than the mean were considered as low.

Statistical analysis

The statistical analysis was conducted using the IBM Statistical Package for Social Science V21. Physical activity, dietary consumption, and academic achievement were analyzed for describing the participants' characteristics. One-way analysis of variance (ANOVA) was used to test for significant differences in lifestyle behaviors, food consumption, and academic achievements. Univariable logistic regression was first used to assess the associations between students' lifestyle behaviors, and their academic achievement. Next, multivariable models were used to adjust for potential confounders and body weight status. All lifestyle behaviors were considered in a full model to assess independent associations between meeting each lifestyle behavior, body weight status, food group consumptions, and academic achievement.

Finally, a series of linear regression analyses were used to examine the difference between the selected courses (Arabic language, English language, math, science, and the total score), physical activity, and nutrition.

All tests were controlled by SES, age, and sex. Also, the effect of the interaction between nutrition and physical activity on academic scores were examined by the linear regression analysis.

Results

A descriptive analysis of study participants indicated that students from grade 5-9 (11-16 years) were selected. The distribution by gender was

47.3% boys and 52.7% girls. The student's sample was 55.1% from public schools and ~45% from UNRWA (Refugee schools). In regards to parent's education, 66% of fathers had less than 12 years of education and 34% had more than 12 years of education. An almost similar trend was found for the mother's education. Parents' education level was very similar between boys and girls. More than half of the students had moderate family economic status (55.1%), while 34.7% and 10.2% had low and high economic status respectively. Students' lifestyle characteristics including physical activity, healthy food consumption, smoking, and BMI are given in table 1. The majority of students found to be physically active in all ages, only 17.1% of students had reported a low level of physical activity. However, more girls had a moderate activity level than boys (60.3%, 52.2%), and boys had higher activity level than girls (38.2%, 16%), respectively. About 43% of students spent 1-2h/day in leisure time activity, while more girls spent <1h/day than boys (35.9%, 27.8%) respectively, and more boys spent >3h/day than girls (29.1%, 20.5%), respectively. About 10% of students were smoking cigarettes or Nargila, and a higher prevalence of smoking was found in boys. The BMI results showed that 5.5% of students underweight, 9.7% were overweight and 4.2% obese. Higher prevalence of overweight and obesity rates was found in girls.

Table 2 presents the relation between academic achievement and the students' intake from different food groups. The high academic achievement group reported higher fruits and vegetable consumption than lower academic achievement groups. On the other hand, the low academic achievement group reported higher soft drink and energy drink consumption.

The adjusted odds ratio (AOR) analysis showed increased odds of high academic achievement in boys and girls who had higher fruits consumption (AOR: 1.1 (0.72-1.68); 1.18(0.81-1.7), respectively, and boys and girls who had higher vegetable consumption (AOR:1.21(0.8-1.82); 1.33(0.93-1.91), respectively. Furthermore, the adjusted analysis indicated a decreased odds in boys and girls of high academic achievement with lower intake of soft drink, beverages (juice with sugar) and energy drink (AOR: 0.79(0.5-1.23); 0.85(0.58-1.27)); (0.99(0.63-1.57);0.76(0.52-1.12));(0.66(0.38-1.15),0.49(0.27-0.89)); respectively. Girls reported a higher intake of sweets and

Table 1: Lifestyle characteristics of schoolchildren by gender.

Characteristics	Category	Boys(n=920)	Girls(n=1025) n(%)	Total
Physical activity	Low activity	89(9.7)	243(23.7)	332(17.1)
	Moderate Activity	480(52.2)	618(60.3)	1098(56.5)
	High activity	351(38.2)	164(16)	515(26.5)
Leisure time activity	<1hour/day	256(27.8)	368(35.9)	624(32.1)
	1-2hours/day	396(43)	447(43.6)	843(43.3)
	>3hour/day	268(29.1)	210(20.5)	478(24.6)
Healthy Food Consumption	Poor	508(55.2)	522(50.9)	1030(53)
	Good	412(44.8)	503(49.1)	915(47)
Smoking	Yes	135(14.7)	56(5.5)	191(9.8)
	No	785(85.3)	969(94.5)	1754(90.2)
BMI	Underweight	62(6.7)	45(4.4)	107(5.5)
	Normal	764(83)	804(78.4)	1568(80.6)
	Overweight	66(7.2)	122(11.9)	188(9.7)
	Obese	28(3)	54(5.3)	82(4.2)

chocolates than boys. The adjusted analysis showed increased odds of high academic achievements in girls and decreased odds in boys who had sweet and chocolate intake. Table 3 presents the physical activity and leisure time activity levels relative to academic achievement. The adjusted analysis showed increased odds of high academic achievement in boys and girls who had high physical activity (AOR: 1.2(0.7-2.1); 1.5(0.9-2.6)), respectively. In addition, results showed increased odds of high academic achievement in boys and girls who had high leisure-time activity, and girls reported higher value than boys, (AOR: 1.3 (0.8-2.0); 1.4 (0.9-2.2), respectively.

Table 4 presents the mean and standard deviation of marks for all groups.The mean marks for all subjects (Arabic, English, math and science) were higher in group 1 (healthy, active, and healthy and active groups) than students in group 2 (unhealthy, non-active, unhealthy and non-active, and overweight and obese groups). Significant differences were found between the two groups.

The linear regression was used to predict the effect of healthy nutrition and physical activity on Arabic, English, math and science achievement scores, controlled by age, sex, parent education, and SES, which is presented in table 5 and 6 The results of linear regression for Arabic language score indicated that healthy

Table 2: Adjusted odds ratio (AOR) and 95% CI for academic achievement in relation to the intake of healthy and unhealthy food items in girls and boys.

	Boys(n=920)			Girls(n=1025)		
	Low academic Achievement (n%)	High academic Achievement	AOR(++)	Low academic Achievement	High academic Achievement(n%)	AOR(++)
Fruits	215(44.2)	224(51.6)	1.1(0.72-1.68)	237(49.3)	288(52.9)	1.18(0.81-1.7)
Vegetables	229(47.1)	230(53)	1.21(0.8-1.82)	236(49.1)	327(60.1)	1.33(0.93-1.91)
Milk & Milk Products	210(43.2)	188(43.3)	0.88(0.58-1.34)	172(35.8)	212(39)	0.93(0.64-1.36)
Sweets & Chocolates	142(29.2)	125(28.8)	0.75(0.47-1.19)	164(34.1)	195(35.8)	1.41(0.96-2.06)
Soft Drinks	193(39.7)	164(37.8)	0.79(0.5-1.23)	173(36)	169(31.1)	0.85(0.58-1.27)
Beverages (juice with sugar)	159(32.7)	159(36.6)	0.99(0.63-1.57)	178(37)	182(33.5)	0.76(0.52-1.12)
Energy Drinks	108(22.2)	66(15.2)	0.66(0.38-1.15)	57(11.9)	31(5.7)	0.49(0.27-0.89)

++Adjusted for BMI, SES and paternal education

Table 3: Adjusted odds ratio (AOR) and 95% CI for high academic achievement in relation to physical activity and leisure time activity by sex.

	Boys (n=920)			Girls(n=1025)		
	Low academic Achievement(n%)	High academic Achievement	AOR(++)	Low academic Achievement	High academic Achievement(n%)	AOR(++)
High physical activity	309(63.5)	306(70.5)	1.2(0.7-2.1)	210(43.6)	231(42.5)	1.5(0.9-2.6)
High Leisure time activity	161(33)	13731.6(31.6)	1.3(0.8- 2.0)	117(24.3)	104(19.1)	1.4(0.9-2.2)

++Adjusted for BMI, SES and paternal education

intake, physical activity, and their interaction were significant predictors of Arabic language score (F (6,1938) =19.9, *p<.01*), with an R2 of 0.058. Healthy intake, physical activity, healthy intake and physical activity, SES, age and sex were significant predictors of Arabic score (HI, t=2.8 *p<0.01*, PA, t=3.2, *p<0.01* [HI x PA], t= 2.5, *p<0.05,* SES, t=5.9, p<0.01, age, t=-5.6, *p<0.01*, sex, t=4.2, *p<0.01*). In addition, the prediction equation for Arabic score (YArabic=60.1+3.3HI+1.5PA+4.5 [HIxPA] +3.6SES-2.8age+3.3sex) indicates the folowing for significant predictors: (1) As healthy intake increased by one unit, the Arabic score increased by 3.3, (2) as physical activity increased by 1 unit, the Arabic score increased by 1.5, (3) as the interaction between healthy intake and physical activity increased by 1 unit the Arabic score increased by 4.5, (4) as the SES increased by 1 unit, the Arabic score increased by 3.6 (5) as age increased by 1 unit, the Arabic score decreased by 2.8 (6) the sex influenced the Arabic score with girls scoring higher by 3.3 than boys.

The results of linear regression for English language score indicated that healthy intake, physical activity, and their interaction were significant predictors of English score (F (6,1938) =18.6, *p<.01*), with an R2 of 0.054.

The healthy intake, physical activity, healthy intake and physical activity, SES, age and sex were significant predictors of English score (HI, t=2.6 *p<0.01*, PA, t=3.3, *p<0.01*, [HI x PA], t= 2.2, *p<0.05*, SES, t=5.9, *p<0.01*, Age, t=-5.3, *p<0.01*, Sex, t=3.8, *p<0.01*). In addition, the prediction equation for English language score (Y English=55.4+3.3HI+1. 7PA+4.2 [HIxPA]+3.9SES-2.9age+3.2sex) indicates the following for significant predictors: (1) As healthy intake increased by one unit, the English score increased by 3.3, (2) as physical activity increased by 1 unit, the English score increased by 1.7, (3) as the interaction between healthy intake and physical activity increased by 1 unit the English score increased by 4.2, (4) as the SES increased by 1 unit, the English score increased by 3.9 (5) as age increased by 1 unit, the English score decreased by 2.9 (6) sex influenced the English score with girls scoring higher by 3.2 than boys. The results of linear regression for math score indicated that healthy intake, physical activity, and their interaction were significant predictors of math score (F (6,1938) =13.3, *p<.01*), with an R2 of 0.038. The healthy intake, physical activity, SES, and age were significant predictors of math score. (HI, t=1.7 *p<0.05*, PA, t=2.8, *p<0.05*, SES, t=5.8, *p<0.01*, Age, t=-5.5, *p<0.01*). In addition, the prediction equation for math score (Ymath=58.7+2.2HI+1.4PA+3.9SES-3age) indicates the following for significant predictors:
(1) As healthy intake increased by one unit, the math score increased by 2.2, (2) as physical activity increased by 1 unit, the math score increased by 1.4, (3) as the SES increased by 1 unit, the math score increased by 3.9 (4) as age increased by 1

	Arabic	English	Math	Science	Total Av. Score
Healthy intake	74.1±16.9 **	70±19**	67.3±18.4*	72.5±18 **	75.9±13.1
Unhealthy intake	70.8±17.7 *	66±19 *	64.9±18.7*	68.9±18.5 *	73.7±12.9
Active	73.7±16.8*	69±18	67.6±18*	72.3±17.1*	75.3±13.1
Non-Active	71.9±17.5 *	67±19	65.5±18.7*	70±18.1 *	73±13.1
Healthy & Active	74.4±17 *	70±18*	68±18.4	73.6±17.1 *	73.6±13.1
Unhealthy & non Active	70±18 *	66±19 *	64.3±18.8*	68±18.9 *	76.5±13.1
Overweight & Obese	70.6±17.3	64±19 *	62.4±18.9 **	67.6±18.3 *	71.7±12.1

Table 4: Means and Standard Deviations of academic achievements for all Groups.
** P<0.05, ** P<0.01*

unit, the math score decreased by 3. The results of linear regression for science score indicated that healthy intake, physical activity, and their interaction were significant predictors of science score (F (6,1938) =14.6, *p<.01*), with an R2 of 0.043. The healthy intake, physical activity, healthy intake and physical activity, SES, age and sex were significant predictors of science score (HI, t=2.6 *p<0.01*, PA, t=3.5, *p<0.05*, [HI x PA], t= 2.5, *p<0.05*, SES, t=5.7, *p<0.01*, Age, t=-3.9, *p<0.01*, Sex, t=2, *p<0.01*). In addition, the prediction equation for science score (Yscience=59.6+ 3.1HI+1.7PA+4.7[HI x PA]+3.7 SES-2.0 age+1.6sex) indicates the following for significant predictors: (1) As healthy intake increased by one unit, the science score increased by 3.1, (2) as physical activity increased by 1 unit, the science score increased by 1.7, (3) as the interaction between healthy intake and physical activity increased by 1 unit the science score increased by 4.7, (4) as the SES increased by 1 unit, the science score increased by 3.7, (5) as age increased by 1 unit, the science score decreased by 2.0 (6) sex influenced the science score with girls scoring higher by 1.6 than boys.

The results of linear regression for total average score indicated that healthy intake, physical activity, and their interaction were significant predictors of total average score (F (6,1938) =16.2, *p<.01*), with an R2 of 0.045. The healthy intake, physical activity, healthy intake and physical activity, SES, age and sex were significant predictors of total average score (HI, t=2.7 *p<0.01*, PA, t=3.5, *p<0.05*, [HI x PA], t= 2.0, *p<0.05*, SES, t=6.6, *p<0.01*, Age, t=-4.0, *p<0.01*, Sex, t=2.5, *p<0.05*).In addition, the prediction equation for total average score (Ytotal Average=66.2+2.4HI+1.3PA+2.8[HI x PA]+3.2 SES-1.5 age+1.6 sex) indicates the following for significant predictors: (1) As healthy intake increased by one unit, the total average score increased by 2.4, (2) as physical activity increased by 1 unit, the total average score increased by 1.3, (3) as the interaction between healthy intake and physical activity increased by 1 unit the total average score increased by 2.8, (4) as the SES increased by 1 unit, the total average score increased by 3.2 (5) as age increased by 1 unit, the total average score decreased by 1.5 (6) sex influenced the total average score with girls scoring

Table 5: Linear Regression of Arabic and English languages scores.

	Arabic Language			English Language		
	B	t	95%CI	B	t	95%CI
Healthy Intake	3.3	2.8**	(-5.18 - 1.83)	3.3	2.6**	(-4.93 - 2.69)
Physical Activity	1.5	3.2**	(0.6 - 2.44)	1.7	3.3**	(0.69 - 2.69)
Healthy and Active	4.5	2.5*	(1 - 8)	4.2	2.2*	(0.39 - 7.99)
SES	3.6	5.9**	(2.44 - 4.85)	3.9	5.9**	(2.64 - 5.26)
Age	-2.8	-5.6**	(-3.79 -1.83)	-2.9	-5.3**	(-3.94 - -1.81)
Sex	3.3	4.2**	(1.77 - 4.84)	3.2	3.8**	(1.54 - 4.88)

** P<0.05, ** P<0.01*

Table 6: Linear Regression of math, science and total average scores

	Math			Science			Total average score		
	B	t	95%CI	B	t	95%CI	B	t	95%CI
Healthy Intake	2.2	1.7*	(-5.43 - 2.22)	3.1	2.6**	(-5.21 - 2.13)	2.4	2.7**	(-3.61 - 1.86)
Physical Activity	1.4	2.8*	(0.41 - 2.41)	1.7	3.5*	(0.75 - 2.67)	1.3	3.5*	(0.57 - 2.01)
Healthy and Active	3.2	1.7	(-0.59 - 7.05)	4.7	2.5*	(1.08 - 8.41)	2.8	2.0*	(0.12 - 5.57)
SES	3.9	5.8**	(2.54 - 5.17)	3.7	5.7**	(2.44 - 4.96)	3.2	6.6**	(2.23 - 4.1)
Age	-3.0	-5.5**	(-4.08 - -1.95)	-2.0	-3.9**	(-3.07 - -1.02)	-1.5	-4.0**	(-2.31 - -0.79)
Sex	1.3	1.5	(-0.41 - 2.94)	1.6	2.0*	(0.02 - 3.24)	1.6	2.5*	(0.36 - 2.75)

** P<0.05, ** P<0.01*

higher by 1.6 than boys. Table 7 presents the linear regression analysis of negative health behaviors with the students' total average score. The results indicated that unhealthy intake (UI), non-active (NA), the interaction between unhealthy intake and non-active (UINA), and the overweight and obese (OO) were significant predictors of total average score (F (7,1937) =14, *p<.01*), with an R2 of 0.05. Unhealthy intake, non-active, unhealthy intake and non-active, overweight and obese, SES, age and sex were significant negative predictors of total average score (UI, t=-3.0 *p<0.01*, NA, t=-2.1, *p<0.01*, [UI x NA], t= -2.3, *p<0.01*, OO t=-3.2, *p<0.01*, SES t=6, *p<0.01*, Age, t=-4.0, *p<0.01*, Sex, t=2.9, *p<0.01*). In addition, the prediction equation for total average score (Ytotal average=72-1.9UI-2NA-2[UIxNA]-3OO+3.1SES-1.7age+1.9sex) indicates the following for significant predictors: (1) As unhealthy intake increased by one unit, the total average score decreased by 1.9, (2) as nonphysical activity increased by 1 unit, the total average score decreased by 2, (3) as the interaction between unhealthy intake and nonphysical activity increased by 1 unit the total average score decreased by 2, (4) as the overweight and obesity increased by 1 unit, the total average score decreased by 3, (5) the SES increased by 1 unit, the total average score increased by 3.1 (5) as age increased by 1 unit, the total average score decreased by 1.7 (6) sex influenced the total average score with boys scoring higher by 1.9 than girls.

Table 7: Linear Regression of unhealthy lifestyle and total average score

	Total average score		
	B	t	95 % CI
Unhealthy Intake	-1.9	-3**	-3.2 - -0.7
Non-Active	-2	-2.1**	-3.9 - -0.1
Unhealthy & non-active	-2	-2.3**	-3.6 - -0.3
Overweight & Obese	-3	-3.2**	-4.8 - -1.1
SES	3.1	6**	2.1 - 4.1
Age	-1.7	-4**	-2.5 - -0.9
Sex	1.9	2.9**	0.6 - 3.2

** P<0.05, ** P<0.01*

Discussion

Our study aimed to identify the links between healthy food consumption and regular physical activity and to identify the significant effect on students' academic achievement among Palestinian schoolchildren. It has been identified that normal level of physical activity and proper nutrition have a significant effect on students' growth, cognitive development and academic achievement. Moreover, it has been found that overweight and obesity have a negative impact on students' mental health and cognitive development. However, in Palestine, few studies assessed the effect of physical activity and proper nutrition on students' cognitive development and academic achievement (Abudayya et al. 2011). To the best of our knowledge, no other studies have examined the association between health behaviors and academic achievement on a national survey among schoolchildren in West Bank. The study demonstrated a strong association between proper food intake and regular physical activity and academic achievement among students in grades 5-9 in the West Bank. Healthy food consumers and physically active students reported a strong association with high academic achievement, which remains highly significant after adjustment with socioeconomic status, age, and sex. The study also shows a negative association between overweight and obesity and sedentary behaviors with academic achievements. This study showed not only proper nutrition and regular physical activity have an association with academic achievement, but also the interaction between healthy food intake, physical activity, parents' education, and socioeconomic status had a strong association with academic achievement.

Our findings recommend that healthy food intake and adequate physical activity may positively affect academic achievement scores. The regression analysis results showed that students who consumed healthy food intake

(fruits and vegetables) had higher scores than students who consumed unhealthy food intake (soft drinks, chocolates, and energy drinks) in the Arabic language, English language, math, science, and the total average score. Furthermore, results showed that students with a high physical activity level reported higher scores than non-active students in all selected courses and the overall average score. On the other hand, unhealthy food consumers, non-active, and overweight and obese students reported lower academic achievement scores in Arabic, English, math, science, and the overall average score. The categorization of food intake, physical activity into healthy and unhealthy food intake, and active and sedentary lifestyle behaviors are consistent with other similar studies (Ramirez and Meyer 2006; Rampersaud et al. 2005; Stea and Torstveit 2014; Bleiweiss-sande et al., 2019; Abudayya et al. 2011). The categorization of academic achievement scores was consistent with the literature (Stea and Torstveit 2014). The study is in accordance with other previously published studies, which have reported the association between nutrition, physical activity, and academic achievement. Some studies focused on meal patterns and academic achievement and focused on the importance of breakfast and the regularity of meal patterns throughout the day (Rathi, Riddell, and Worsley 2017; Montazerifar, Karajibani, and Dashipour 2012; Vadiveloo, Zhu, and Quatromoni 2009; Bleiweiss-sande et al., 2019).

To best to our knowledge, no other studies examined the relationship between specific food intake and academic achievement among schoolchildren in West Bank. Results from our study showed increased odds of high academic achievement in boys and girls who had a higher intake of fruits and vegetables. In contrast, the results showed a decreased odds of high academic achievement in boys and girls who had a high intake of soft-drink, sugar, and energy drink. The study results are consistent with other similar studies that reported increased odds of high academic scores in student who had high fruit and vegetable consumption (Hou et al. 2020; Kristo et al. 2020; Bleiweiss-sande et al., 2019). Our study results are also consistent with other studies that reported that high consumption of salty snacks and soft-drinks is associated with low academic achievement. However, the study is inconsistent with other studies which reported lower odds of academic scores for girls with high sweet and chocolate consumption (Asigbee et al., 2018; Kim et al., 2003; Castelli et al., 2015; Vadiveloo, Zhu, and Quatromoni 2009; Stea and Torstveit 2014). Other studies reported that lower intake of fruits and vegetables and a high intake of sweets, soft drinks and energy drinks was associated with lower math score (Vadiveloo, Zhu, and Quatromoni 2009; Rampersaud et al. 2005; Rathi, Riddell, and Worsley 2017; S. Y. Kim et al. 2016; Stea and Torstveit 2014; Abudayya et al. 2011). Our study also examined the effect of physical activity and leisure time activities on academic achievement scores. The results showed a positive association between high physical activity, lower leisure time activity, and academic achievement in both boys and girls. The results are consistent with other studies that revealed a positive association with academic achievement (Ruiz et al. 2010; Rodríguez García et al. 2014; Armstrong and Welsman 2006; Moral-García et al. 2020; Hou et al. 2020; Janssen 2012; Stea and Torstveit 2014; Taras 2005). The Spanish and Norwegian studies reported that the high academic score had a significant association with physical activity and leisure time activity (Stea and Torstveit 2014; Rodríguez García et al. 2014). Our results reported that physical activity was positively associated with academic achievement in boys, and the leisure time activity was positively associated with academic achievements in girls, which is consistent with multiple studies (Desai et al. 2015; Taras 2005; Asigbee et al., 2018). The sex-specific association might be explained by the differences in the interest of boys and girls in physical activity, and also might be due to cultural issues and the lim-

ited available after school activities for girls. The linear regression analysis examined the effect of food intake, physical activity, and the interaction between food intake and physical activity on the academic achievement score of the Arabic language, English language, math, science, and the total average score.

The results showed a significant association between healthy intake and physical activity and the academic achievement, for example, for math achievement scores, healthy intake increased the academic scores by 2.2, the physical activity increased the academic scores by 1.4, and the interaction between health intake and physical activity increased the academic scores by 3.2. The study result is in accordance with another study that showed a significant increase in academic achievement scores of reading, math, and science scores with the increase of healthy nutrition and physical activity (Janssen 2012; Stea and Torstveit 2014; Asigbee el al., 2018). On the other hand, the linear regression analysis examined the effect of unhealthy behaviors on the students' overall average academic score. The results showed that if unhealthy food consumption increased by one unit the scores decreased by 1.9, and the decrease in physical activity by one unit will decrease the scores by 2, the interaction between unhealthy and low physical activity increase by one unit the scores will decrease by 2, and the increase of overweight and obesity by one unit will decrease the academic scores by 3, the results are in accordance with other previous studies (Asigbee et al., 2018). Furthermore, the results of this study are mostly in line with the results from previous studies that examined the effect of health behaviors on academic scores among schoolchildren (Stea and Torstveit 2014; Moral-García et al. 2020; Kim et al., 2003; Kristo et al. 2020; Faught et al., 2017). There are some limitations to the present study. First, our findings were limited to the West Bank population and don't include students from Gaza. Our results would be strengthened by studying a homogeneous national sample from all Palestinian cities.

Second, the study did not examine the eating habits and meal patterns throughout the day time and doesn't include the breakfast pattern. However, the study strengthened by examining specific food intake, the variety of physical activities, and leisure time activities. Another possible limitation, that the self-reported nature of health behaviors can be prone to bias. Despite these limitations, our study is strengthened by using a representative weighted sample of the West Bank population and conducted by well-trained research assistants under the direct supervision of the school health behavior department at the Ministry of Education.

Conclusion

In conclusion, the results of the present study indicated that the academic achievement of Palestinian schoolchildren was strongly associated with regular physical activity and healthy food intake. The average consumption of fruits and vegetables and the average level of physical activity are relatively important for improving students' academic achievement scores. Lower academic scores were found among students with low physical activity rates, high unhealthy food intake, and obesity.

Based on the study finding, future intervention studies should investigate the combined effect of nutrition and healthy lifestyle, on cognitive development and academic achievement. Furthermore, the study supports the implementation of a school health behavior program that promotes a healthy lifestyle which may lead to better academic achievement. Specifically, school educators may consider developing new courses that will improve students' knowledge of adopting a healthy lifestyle.

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Ethics declaration

The study received ethical approval from the Ministry of Education and the Al-Quds University Institutional Review Board (IRB). Parental consent approval to participate in the survey was obtained from all participants. The data were collected, entered, and cleaned by trained research assistants under the supervision of the Ministry of Education and Al-Quds Nutrition and Health research institute (ANAHRI) at Al-Quds University.

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