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Article

The Integral Core Nutrient Matrix Causing Uterine Fibroid and the Associated Biochemical Roots

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Abstract: Uterine fibroid (UF) is a tumour in some parts of the uterus, which introduces health challenges or death due to failed surgery among women globally. This study was designed to ascertain the involvement of micro-nutrients, inflammation, and antioxidant enzymes in the UF development to gain further insights and provide a strategy for managing the disease. One hundred ninety reproductive-aged women were recruited and classified equally into case and control subjects. The supernatant obtained from excised tissues from the fibroid and the normal samples from the adjacent myometrium were assessed for the selected biochemical parameters with standard methods. The levels of vitamin A and sodium between 26-35 years; vitamins D, E, zinc, and selenium between 46-55 years; and vitamin E at 56 years and above significantly decreased ($p<0.05$). Interleukin-2 (IL-2) level significantly increased ($P < 0.05$) among the case between 36-45 years. An increase in the activity of glutathione-s-transferase and the reduction in glutathione peroxidase activity and vitamin A level in the uterus between 26-45 years were the most pronounced significant findings ($p<0.05$) recorded. Prolonged vitamin A deficiency coupled with excess sodium salts facilitating inflammation induced by IL-2 are critical factors for UF development.

Keywords: Disease; fibroid; micronutrients; inflammation; antioxidants; cytokines; diets

1. Introduction

A healthy diet for the general health status of humans is unarguable of importance [1]. A healthy diet comprises fruits and vegetables, vitamins, micronutrients from food intake, and mineral supplements [1]. The influence of nutritional composites on hormonally related diseases such as endometrial cancer, breast cancer, and uterine fibroid (UF) is vital to tumour development [1-3]. The diet has been implicated as one of the risk factors of uterine fibroid, just like breast cancer, because both diseases are hormonally inclined and possess similar risk factors like age and race [4]. Micronutrients, vitamins, and minerals are derived from diets which signify their importance in metabolism in the body as some of them serve as pro-hormones and co-enzymes [5]. He and his colleague in China evaluated the effect of diet on the pathogenesis of uterine fibroid. Diet was reported as one of the contributors to the development of leiomyomas [6]. The correlation between fibroid, fruits, and vegetables was also reported in a study in Italy. The conclusion affirmed the importance of a healthier diet and lifestyle habits based on a higher intake of vegetables, fruits, and fish as preventive measures against different health issues [7,8].

Studies have shown that fruits and vegetables consist of antioxidants and various phytochemicals that can inversely reduce the risk of uncontrollable cell growth [9]. In contrast, intake of red meat, ham, and burgers were linked to UF development [10]. There is a conflicting explanation of the impact of some macro and micronutrients on UF development. Wise, Radin, Palmer, Kumanyika, Boggs and Rosenberg [9] claimed no association between vitamin E, folate, and UF. His team also showed no correlation between vitamin

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A, B_6, and B_{12}. However, Martin, et al. [11] showed the inhibitory role of butyric acid, calcium, and vitamin A in cell proliferation, though the vitamin A from the study was from an animal source. Soy product is rich in isoflavones - a compound made of phytoestrogens and believed to decrease UF risk [12]. Conversely, previous studies found no correlation between fibroid risk and soy compounds [6,12,13]. However, experimental studies on UF cell lines validated the role of flavonoids on cell proliferation and apoptosis even though the flavonoid was from the barbed skullcap plant with the botanical name *Scutellaria barbata* D [14]. Similarly, epigallactocatehin gallate (EGCG from green tea) showed its inhibitory potency in proliferation and induced apoptosis in UF [15,16].

UF is a common disease requiring gynaecology attention in both developed and developing countries. UF is most prevalent among black women than among the women of Caucasian and Asian origin [17]. Although many risk factors have been established as the initiator and promoters of the tumour, however, modifiable lifestyle factors could also partake in the progression and development of UF, as was the case in other diseases such as hypertension, obesity, inflammation, and hypercholesterolemia [1]. The impact of UF on the quality of life of many women requires efficient treatment plans by considering modifiable lifestyle factors as preventive and treatment methods [18]. Efforts in investigating the significance of a healthy lifestyle, suitable dietary measures, and hygiene might go a long way in finding a lasting solution to UF lesions. Fortunately, a recent study revealed the interaction of gene-nutrient to health management and disease prevention [18].

No literature has been able to identify the authentic influencer of UF growth and development. However, several pathogenetic $\alpha\alpha$s have been implicated in the growth and development of leiomyoma. These pathogenetic factors include hormones, genetics, growth factors, cytokines, chemokines, diet, physical activities, oxidative stress, and other environmental issues. Even the initiators or promoters of the tumour remain completely uncleared. Nevertheless, the percentage of hysterectomies and myomectomies performed justify that the tumour is a public health issue [19]. There are increasing efforts to examine the influence of micronutrients on the growth and proliferation of UF [18,20]. None of these previous studies engaged the uterus in humans. Therefore, this study aimed to determine the influence of the micronutrient, antioxidant status, and inflammation on UF development directly from human, particularly the uterus tissue.

2. Materials and Methods

2.1. Study Location

The study location for the clinical aspect of the study was University College Hospital (UCH) Ibadan in Oyo State, geographically located at 7.3565° N, 3.8748° E and Adeoyo Marternity and Teaching Hospital, Ibadan, Oyo State, located geographically at 7.3638024^0 S, 3.8646299^0 W. Blood samples were collected from ultrasonically screened and confirmed Age-matched non-UF subjects (control) at Nissi Doppler Ultrasound diagnostic center, opposite Adeoyo Maternity and Teaching Hospital. The samples collected were analysed at the Biochemistry Laboratory of Covenant University, Sango-Ota in Ogun-State, located at 6.6726° N, 3.1612° E. All sites are located in Nigeria.

2.2. Sample Size Determination

The sample size was determined by considering the already quoted 6.58 % of the recorded prevalence of UF in Ile-Ife, Southwest Nigerian women [21]. The following general formular 1 for calculating sample size was followed [22,23].

$$Z_1\text{-}\alpha/2^2 \times P(1- P)/d^2 \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \text{formular}$$

Where $Z_1\text{-}\alpha/2^2$= 1.96 (standard normal variant corresponding to the level of confidence interval at 5 % error.

P = Previous given prevalence= 6. 58= 0.0658 [21]

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d = Precision representing effect size =5 % [22]

Therefore, $(1.96)2\ 0.0658(1-0.0658)/(0.05)2 = 94.5$, approximately 95.

This value (95) was used as the sample size for the study.

2.3. Inclusion and Exclusion Criteria

The criteria considered were declared as follows:

Inclusion Criteria: Females who have attained menarche (age ≥14 years) with at least one ultrasonographically-diagnosed uterine fibroid of a minimum diameter of 2 cm who had been scheduled for surgical treatment of the fibroids and age-matched fibroid-free participants (control) excluded by ultrasound scans were included in the study.

Exclusion criteria: Women with chronic systemic disease, malignancy, pregnancy, vitamin D replacement, lactation, abortion, or those on oral contraceptive/hormonal agents in the past three months were excluded from the study.

2.4. Ethical Application Process and Informed Consent

The ethical approval was obtained from the Covenant Health Research and Ethics Committee (CHREC) and the joint University of Ibadan/University College Hospital Ethical Review Committee with the approved number CHREC/38/2020, and UI/EC/19/0268, respectively. The recruited participants were made to sign the informed consent after the study objectives had been fully explained to them.

2.5. Experimental Design

2.5.1. Participants Recruitment Process.

One hundred and ninety (190) women participated in this study, ninety-five (95) clinical cases (fibroid patients) and ninety-five (95) control (age-matched non-fibroid patients), all confirmed by ultrasonography. Each participant signed the informed consent (Appendix S1), and then the predesigned questionnaire was also administered to each of the subjects (Appendix S2). The questionnaire consists of socio-demographic, anthropometric, and clinical information. Ultrasound scanning of the uterus of the subjects was also performed to confirm the presence or absence of uterine fibroids. The biological specimens obtained were UF tissue, normal myometrial tissue and venous blood samples.

2.5.2. Biological Sample Collection

The obtained biological specimens (blood and tissues) were classified as the case (UF samples) and control (non-UF samples). Grouping was done based on their age and UF status. The blood samples were taken from (both UF patients and non-UF participants) at the two-hospital used for the study. An intra-surgical fibroid tissue sample and non-fibroid tissue from the adjacent side of the myometrium were collected from the UF participants. The excised tissues were rinsed with normal saline and frozen at -20 ^{0} C for biochemical study and later prepared for further analysis.

2.5.3. Preparation of Biological Samples

2.5.3.1. Preparation of Serum from the Blood Sample

The blood obtained was portioned into two. A portion of 8 mL of the blood was collected inside the non-anti-coagulant tube (serum separator tube). This portion of the blood was allowed to clot and centrifuged to obtain serum using the standard procedure. The serum harvested from this was kept at -80 °C for biochemical analysis. The other 2 mL of the whole blood was collected inside the EDTA bottle for haematological analysis.

2.5.3.2. Preparation of Fibroid Tissues Homogenates

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A portion of 5 g of the freshly excised fibroid tissues collected was divided into three sections. A portion of the tissue (1 g) was kept in a 10 % formalin solution for histological study. Another portion of the excised tissue (2 g) was rinsed with normal saline and preserved by freezing at -80 °C. Later, this was homogenised in 10 mL phosphate buffer (0.1 M, pH 7.4). The slurry mixture was spun at 4,000 × g for 10 minutes as described by Afolabi, *et al.* [24] with slight modifications before storing the supernatant at -20 °C for further biochemical analysis. Total RNA was isolated from the remaining 2 g excised tissues in RNAlater® using an RNA extraction kit (Trizol reagent, Invitrogen). The extracted RNA was kept at -80 °C for molecular analysis. This same process was used for the normal tissue (adjacent myometrial tissues).

2.5.3.3. Grouping of the Participants

The experimental design for this study was in two phases: - a case-control model of UF patients and non-UF participants. In the model, all entire UF-patients and non-UF participants were considered as two entities (case and control). Then, the second phase is a case-control stratified further by age model that resulted in the following five groups of case and controls each: (16-25), (26-35), (36-45), (46-55), and (56 & above). However, there was no age-matched for group A control (16-25), leaving us with four case and control groups, as presented in Table 1. Our choice of 10 as the class interval was arbitrary to minimise the number of classes to a manageable size of only five groups.

Table 1. Age distribution.

S/N	Groups	Age distribution	Case (UF samples)	Control (non-UF samples)
1	A	16-25 years	0	19
2	B	26-35 years	24	42
3	C	36-45 years	48	19
4	D	46-55 years	19	13
5	E	56 & above	4	2

2.6. Biochemical parameters monitored during experimentation

2.6.1. Determination of Micronutrients (Vitamins and Mineral Element) Levels

ELISA kits were employed to determine vitamin A (Kit Catalog no: E-EL-0135), D (Kit Catalog no: VD220B) and K (Kit Catalog no: E-EL-0019), and the colourimetry method was used for vitamin C (Kit Catalog no: E-BC-K034-S) and E determination (Kit Catalog no: E-BC-K033-S), following the instructions stated by the manufacturer (Elabscience Biotechnology Inc, Houston, USA) as illustrated in 2.6.1.1- 2.6.1.5. The mineral elements in the samples were quantified using Atomic Absorption Spectroscopy (AAS), as illustrated in 2.6.2.

2.6.1.1. Procedure for the Determination of Vitamin A (VA)

The vortexed mixed reagents was mixed with 50 µL of the sample as specified, incubated for 15 minutes at 37 ^{0}C, while evading light, before stoping the reaction. The microplate reader was preheated for 15 minutes, and the absorbance was read at 450 nm. The absorbance was obtained in duplicate, and the concentration of vitamin A was extrapolated from the prepared graph.

2.6.1.2. Procedure for the Determination of Vitamin D (VD)

The vortexed mixed reagents was mixed with 10 µL of the sample as specified before stoping the reaction. The absorbance was read in duplicate at 450 nm, and the concentration of vitamin D was extrapolated from the prepared graph.

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2.6.1.3. Procedure for the Determination of Vitamin K (VK)

The vortexed mixed reagents was mixed with 50 µL of the sample as specified before stoping the reaction. The absorbance was immediately read at 450 nm. The equivalent vitamin K concentration for the duplicate readings for both standard and samples were extrapolated from a prepared standard graph. The actual concentration is the calculated concentration multiplied by the dilution factor.

2.6.1.4. Procedure for the Determination of Vitamin C (VC)

The vortexed mixed reagent was mixed with 0.15 mL of the sample, and allowed to stand for 15 minutes at room temperature before centrifuging at 2000 g for 10 min. The supernant obtained was equally treated as specified, vortexed and incubated at 37 ^{0}C in a water bath for 30 min. The spectrophotometer was set to zero with double distilled water, and the absorbance (OD) was taken at 536 nm using a 1 cm optical path cuvette. The concentration of the equivalent vitamin C was calculated using the following formular 2.

VC content (µg/mL) = $\Delta A_1/\Delta_2$ x C x F x 4…… formular 2.

Where ΔA_1= OD Sample- OD Blank, ΔA_2: OD Standard – OD Blank, C: Concentration of Standard (6 µg/mL), F= Dilution factor of the sample before the test, 4 = Dilution factor of sample preparation (4 times).

2.6.1.5. Procedure for the Determination of Vitamin E (VE)

The reagents were mixed with serum sample (0.3 mL) as specified, and vortexed for 10 seconds. Then, 1 mL of absolute ethanol was added, vortexed, and allowed to stand at room temperature for 2 minutes. The spectrophotometer was standardised to zero with absolute ethanol, and a sizeable 0.5 cm optical cuvette was used to determine the OD values of each tube at 533 nm. The concentration of the equivalent vitamin E was calculated using the following formular 3.

VE (µg/mL) = $OD_{sample} – OD_{blank}$ x C x f x 2*/ $OD_{Standard} - OD_{blank}$ .. formular 3.

Where C = Concentration of standard (10 µg/mL), f = dilution factor of the sample before the test, and 2*= the volume of the standard is 0.6 mL, the volume of sample is 0.3 mL (the sample was condensed twice).

2.6.2. Procedure for the Determination of Minerals Elements

The instrument first processed the zinc/copper-containing sample to generate a ground-state atom as a vapour within the light path of the device. This process, called atomisation, was done using flame-atomic absorption spectrometry (FAAS). The atoms of the elements were vaporised and atomised in the flame. The atoms then absorbed the light at a characteristic wavelength. The radiation of an appropriate wavelength produced by the lamp was absorbed by free atoms of the sample while passing through the flame. A photo-detector read-out system measured the absorbed energy of each element. The amount of energy absorbed is proportional to the concentration of the element in the sample.

2.6.3. Antioxidant Enzymes

2.6.3.1. Procedure for the Determination of Glutathione Peroxidase (GPx) in Serum and Tissue Samples

Analysis of glutathione peroxidase was determined using the method of Rotruck, *et al.* [25]. The activity of GPx is calculated using the following formular 4.

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GPx activity (umol/mg sample) = OD_{412} x TV x df/6.22 x 10^3 x EV .. formular 4

Where; OD_{412} = Absorbance at 412nm, TV = Total volume, df = dilution factor, EV= Enzyme volume and Extinction coefficient= 6.22 $X10^3$.

2.6.3.2. Procedure for the Determination of Glutathione (GSH)

The glutathione level in serum and tissue homogenate was determined using Moron's method modified by Nzekwe, *et al.* [26]. Absorbance was measured at 412 nm against the blank made of distilled water instead of the samples. The concentration of GSH in µg/GSH/g sample that is present in the samples was calculated using the following formular 5, and expressed as.

GSH content = OD_{sample} x 10 x (4.7/2) x 30.7 / ∑412 x weight of sample..… formular 5

2.6.3.3. Procedure for the Determination of Glutathione-S-Transferase (GST) in Serum and Tissue

Glutathione-S-transferase activity in the serum and tissue homogenates was determined as described by Habig, *et al.* [27]. Absorbance was read at 340 nm wavelength for each sample against a blank, and the corresponding GSH activity was calculated using the following formular 6.

GST Activity = (OD_{Sample} – OD_{Blank})/9.6 x 1/Weight of sample in 0.5 mL extract ..… formular 6.

2.6.3.4. Procedure for the Determination of Catalase (CAT) Activity in Serum and Tissue Sample

The determination of CAT was done by employing the method of Beutler [28]. The absorbance read at zero minutes and 1 minute at 240 nm. The corresponding CAT activity was calculated using formular 7.

CAT (mmol/min/g sample) = ΔA/min x VT/∑ x wt ……. formular 7.

ΔA = Change in absorbance, VT = total volume, ∑ = Molar extinction, VS =Sample volume, and wt = weight of samples

2.6.3.5. Procedure for the Determination of Superoxide Dismutase (SOD) in Serum and Tissue Samples

The SOD activity was conducted following the method of Misra and Fridovich [29]. Absorbance was read at 420 nm, and the equivalent SOD activity was calculated using the following formular 8.

SOD (mU/mg sample) = ΔA/min x VT(X)/∑ x VS x wt ……. formular 8.

Where; ΔA = Change in absorbance, VT = Total volume, ∑ = Molar extinction, VS =Sample volume, and wt = weight of samples.

2.6.4. Determination of Inflammatory Cytokines

The following are the procedure for each of the selected inflammatory biomarkers.

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2.6.4.1. Procedure for the Determination of Serum Interleukin -1Alpha (IL-1 alpha) and Serum Interleukin -2Alpha (IL-2 alpha)

IL- 1α (Kit Catalog no: SEKH-0001) or IL- 2α (Kit Catalog no: SEKH-0008) in serum samples was measured using the commercially available ELISA kits, and following procedure provided by the manufacturer (Beijing Solarbio Science & Technology Co., Ltd., China). The kit components and samples were brought to room temperature (25 ^{0}C). A portion of 100 µL of samples was added to each well and incubated for 90 minutes at 37 ^{0}C. The absorbance was taken at 450 nm within 5 minutes. The absorbance for the blank was subtracted from the readings of the absorbance obtained for the duplicate samples. Then, the equivalent concentrations of the interleukins were extrapolated from a prepared standard curve.

2.6.4.2. Procedure for the Determination of Serum CoX-1, CoX-2, and TNF-α in the Sample

CoX-1 (Kit Catalog no: PRS-00487hu; PARSBIOCHEM), CoX2 (Kit Catalog no: SEKH-0413), and TNF-α (Kit Catalog no: SEKH-0047) were measured using the commercially available ELISA kits, and following procedure provided by the manufacturer (Beijing Solarbio Science & Technology Co., Ltd., China). However, manufacturer for the CoX-1 was Nanjing Pars Biochem CO., LTD., China. The appropriate reagents were mixed with sample diluent (40 µL) as specified, and the reaction stopped after evading light for 15 minutes. The colour change was from blue to yellow, and absorbance was quickly taken within 15 minutes at 450 nm. The concentration of the appropriate inflammatory indices that is present the serum was extrapolated from the standard curve generated by plotting the absorbance against the given concentrations.

3. Results

The biological specimen (blood, serum and tissue) from UF subjects and another specimen (blood and serum) from non-UF participants were obtained and grouped into the case and control design model for the first phase. These results were further classified based on age (Case-control Stratified by Age Design**)** for the second phase. The excised tissue samples were UF tissues representing the experimentation, and the adjacent normal myometrial tissues serving as the control. There were no recorded samples for the UF (case) under 26 years, indicating the occurrence of UF is a possible rare phenomenon in that age group within the period of study (Table 1). The levels of minerals in the uterus of healthy women and fibroid women in Nigeria were elucidated, and the pattern of the effects of the micronutrients on fibroid development across age groups was examined for the first time. To the best of our knowledge, this study provides the mineral nutrients status in the uterus of humans for the first time.

3.1.1. Socio-demographic Characteristics of the Participants

Table 2 shows the characteristics of both fibroid and non-fibroid participants based on their tribes, place of abode, educational background, and vocation.

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Table 2: Sociodemographic Parameters

		Groups				Total	
		Case		**Control**			
		Count	%	Count	%	Count	%
Ethnicity	Yoruba	77	81.05	86	90.53	163	85.79
	Hausa	1	1.01	1	3.09	2	1.05
	Ibo	8	8.08	3	3.12	11	5.79
	Others	9	9.09	5	5.15	14	7.37
Residence	Ibadan	19	20.00	26	27.37	45	23.68
	Lagos	45	47.37	39	41.05	84	44.21
	Ogun	26	27.37	21	22.11	47	24.74
	Others	5	5.26	9	9.47	14	7.37
Highest Education	None	0	0.00	0	0.00	0	0.00
	Primary	5	5.26	6	6.32	11	5.79
	Secondary	20	21.05	30	31.58	50	26.31
	Tertiary	70	73.68	59	62.11	129	67.90
Occupation	None	3	3.16	2	2.11	5	2.63
	Apprentice	0	0.00	1	1.05	1	0.53
	Business	39	41.05	40	42.11	79	41.58
	Civil	29	30.53	20	21.05	49	25.79
	Retired	1	1.05	2	2.11	3	1.58
	Others	23	24.21	30	31.58	53	27.90

A total of 190 participants, comprising 95 fibroids (cases) and 95 non-fibroid (controls), were included in the study. The majority of the study respondents were of Yoruba (85.79 %) tribe. Most study participants reside in Lagos, Nigeria (44.21 %). Moreover, most of them had tertiary education (67.90 %) and were businesswomen (41.58 %).

3.1.2. Vitamins Concentrations in UF and non-UF Participants

Figure 1 depicts the concentrations of selected vitamins in the UF (case) and non-UF (control) participants. A significant ($P < 0.05$) decrease was observed in the concentration levels of vitamins A, D, and E in UF patients compared to non-UF. In contrast, a significant ($P < 0.05$) increase was observed in the concentration of vitamin K in UF compared to the non-UF. There is no significant difference in the concentration of vitamin C in both UF and non-UF participants. Table 3 shows the concentrations of the examined vitamins in case-control stratified by age design. There was a significant ($P < 0.05$) decrease in the mean concentration of vitamin A in groups B and C in the UF patients. There was a substantial increase in the mean concentration of vitamin K in group C of myomas compared to the control. In contrast, there was a significant ($P < 0.05$) decrease in the mean concentration of vitamin D in groups D and vitamin E in groups D and E in UF patients compared to their respective age-matched non-UF participants.

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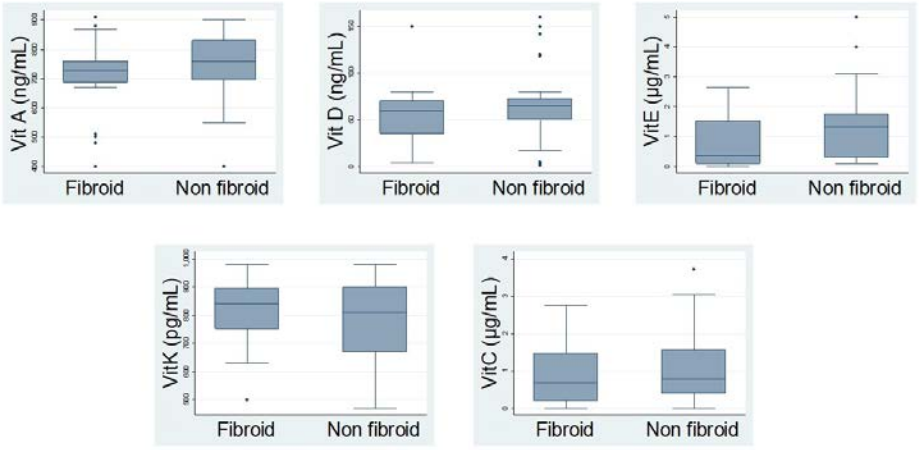

Figure 1: The concentrations of selected vitamins (vitamin A, D, E, K and C) in UF and non-UF Participants. **Keys** - Vit A: Vitamins A, Vit D: Vitamin D, Vit E: Vitamin E, Vit D: Vitamin D, Vit C: Vitamin C.

3.1.3. Concentrations of Micronutrient Elements.

Figure 2. indicates the concentration of micronutrient elements (Na, Ca, Mg, K, Fe, Zn & Se) in the UF and non-UF of the study population. There was a significant ($P < 0.05$) decrease in selenium concentration in UF patients compared with the non-UF participants. Table 4 shows the micronutrient concentration in UF and age-matched non-UF in case-control stratified by age design. There was a significant ($P < 0.05$) decrease in the means concentration levels of Na in UF patients of group B. In contrast, a significant ($P< 0.05$) increase in concentration levels of Na, Mg, Fe and Zn in group C in the UF patients compared to their respective control was observed. There was a significant ($P < 0.05$) reduction in the mean concentration levels of Se and Ca in groups D and E, respectively, in UF patients compared to the non-UF participants. There was a deficiency in vitamin D between the age of 36-45 years, which was not significant ($P>0.05$). This age group is still within the reproductive age. There was a conspicuous rapid increase in Na^+ from the age group of 25-30 years to between 36-45 years, which could facilitate uterine fibroid development. The trend continued at an insignificant level between 46 - 55 years.

Table 3. Concentration of Vitamins in Myomas and Age-matched Non-myomas Groups.

		Vit A (ng/ mL)	Vit D (ng/mL)	Vit E (µg/mL)	Vit K (pg/mL)	Vit C (µg/mL)
B (26-35)	Case	693.077 ± 28.89	50.431 ± 7.79	0.654 ± 0.18	838.85 ± 23.55	1.016 ± 0.15
	Control	753.54 ± 25.84	53.225± 6.94	1.071 ± 0.15	830.500 ± 28.59	0.923 ± 0.25
	P-values	0.042*	0.729	0.082	0.803	0.716

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C (36-45)	Case	678.462 ± 21.32	38.846 ± 4.01	0.827± 0.11	817.231 ± 20.82	0.995 ± 0.17
	Control	742.23 ± 12.73	45.400 ± 4.59	0.963 ± 0.13	699 231 ± 25.56	1.390 ±0.22
	P-value	0.029*	0.408	0.562	4.2×10^{-4}*	0.119
D (46-55)	Case	752.300 ± 14.82	68.700 ± 1.48	0.680 ± 0.16	800.000 ± 16.70	0.620 ± 0.15
	Control	771.300 ± 17.85	95.800 ± 7.80	1.671 ± 0.24	826.000 ± 26.14	0.945 ± 0.11
	P-value	0.565	0.003*	2.8×10^{-4}*	0.487	0.259
E (56 & Above)	Case	727.500 ± 9.33	52.250 ± 7.90	0.844 ± 0.30	857.875 ± 20.60	0.860 ± 0.17
	Control	740.000 ± 44.32	80.250 ± 8.47	2.363 ± 0.56	782.500 ± 56.91	1.147 ± 0.40
	P-value	0.811	0.051	4.2×10^{-4}*	0.203	0.527

Values are expressed as mean ± Standard error of the means (SEM). *: indicate a significant difference between the UF and Non-UF. Vit A: Vitamin A, Vit D: Vitamin D, Vit E: Vitamin E, Vit K: Vitamin K, Vit C: Vitamin C.

Figure 2. The concentration of micronutrient elements in both UF and non-UF. **Keys** - Na: sodium, Ca: calcium, Mg: magnesium, K: potassium, Fe: iron, Zn: zinc and Se: selenium.

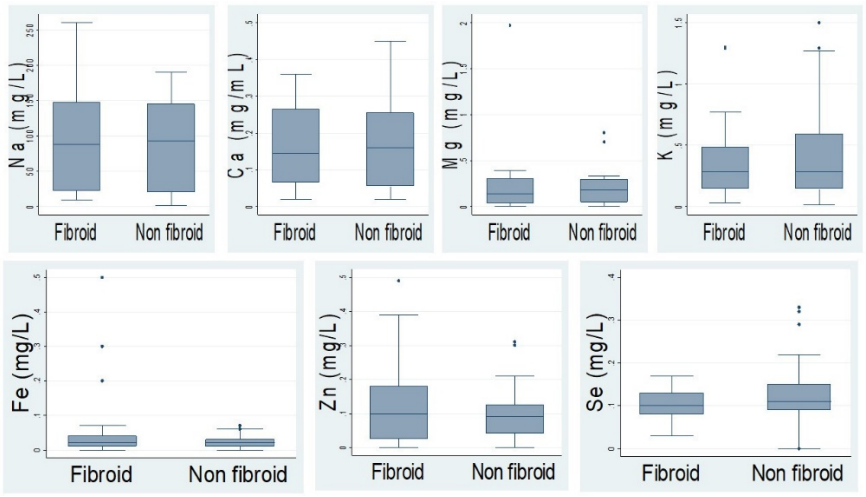

Table 4: Concentration of Micronutrient elements in UF and Age-matched non-UF Participants

Groups	Case	Na (mg/mL)	Ca (mg/mL)	K (mg/mL)	Mg (mg/mL)	Fe (mg/mL)	Zn (mg/mL)	Se (mg/mL)
B 26-35	Case	93.600±3.25	0.243±0.15	0.348±0.02	0.263±0.02	0.034±0.00	0.174±0.014	0.109±010
	Control	135.000±7.03	0.292±0.24	0.404±0.04	0.362±0.05	0.0134±0.00	0.140± 0.02	0.107±0.01
	P-values	4.6×10^{-5}*	0.138	0.376	0.166	0.567	0.115	0.859
C 36-45	Case	198.800±9.36	0.239±0.12	0.678±0.07	0.435±0.12	0.176±0.04	0.194±0.03	0.091±0.01
	Control	122.400±8.76	0.194±0.01	0.768±0.08	0.229±0.02	0.026±0.00	0.126±0.01	0.103±0.00
	P-value	6.4×10^{-13}*	0.165	0.152	0.003*	5.7×10^{-9}*	1.2×10^{-3}*	0.418

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D 46-55	Case	18.772±2.11	0.046±0.004	0.134±0.01	0.016±0.00	0.008±0.00	0.018±0.00	0.100±0.01
	Control	18.248±0.75	0.056±0.01	0.137±0.01	0.0436±0.01	0.015±0.00	0.044±0.00	0.185±0.02
	P-value	0.949	0.765	0.953	0.695	0.760	0.206	7.8×10^{-7}*
E 56 & above	Case	16.140± 0.84	0.270±0.12	0.101±0.01	0.040±0.00	0.030± 0.00	0.021±0.00	0.136±0.01
	Control	12.260±0.58	0.032±0.01	0.038±0.01	0.030±0.00	0.042±0.01	0.027± 0.00	0.140±0.01
	P-value	0.764	7.1×10^{-9}*	0.525	0.927	0.717	0.855	0.864

Values are expressed as mean ± Standard error of the means (SEM)) for UF and non-UF.

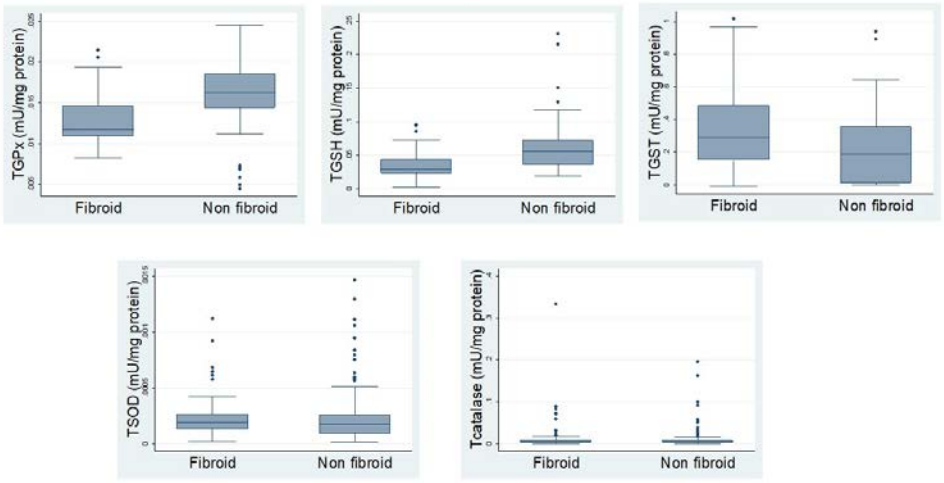
*: signifies significant difference.

Na: sodium, Ca: calcium, K: potassium, Mg: magnesium, Fe: iron, Zn: zinc and Se: selenium.

3.1.4. Concentrations of Tissue Antioxidant Markers in UF and non-UF

Figure 3 depicts the concentrations distribution of the antioxidant enzymes and molecules in the tissue of UF and non-UF participants. There was a significant ($P < 0.05$) decrease in the concentrations of TGPx and TGSH in the myomas group compared to non-myomas groups. In contrast, a substantial ($P < 0.05$) increase in the concentration of TGST was observed in the myomas group compared to the non-myomas group. Table 5 depicts the mean concentrations of antioxidant enzymes in tissues of UF and non-UF participants in case-control stratified by age design. There was a significant ($P < 0.05$) decrease in GSH in groups B, C and E and group B in GPx of the UF groups compared to their respective controls. There was a substantial ($P < 0.05$) rise in the antioxidant enzyme concentration levels in groups B and C GST.

Figure 3. The tissue concentrations distribution of antioxidant enzymes in myomas patients and non-myomas participants. Keys - TGPx: glutathione peroxidase, TGST: glutathione-S-transferase, TGSH: reduced glutathione, TCAT: catalase and TSOD: superoxide dismutase.



3.1.5. Concentrations of Serum Antioxidant Markers

Figure 4 displays the concentrations distribution of serum antioxidant enzymes and molecules in UF patients and non-UF participants. A substantial ($P < 0.05$) increase in the SGPx and SGST concentrations levels in UF compared with the control was detected. Table 6 shows the result of the antioxidant enzymes in the serum of UF and age-matched

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non-UF women in case-control stratified by age design. A significant ($P < 0.05$) decrease was seen in groups B, C and E of GPx. Similarly, there was a substantial ($P < 0.05$) reduction in GST of group B and SOD of group E in the case compared to their respective controls.

Figure 4. The concentration distribution of antioxidant enzymes in the serum of supernatants of UF

Table 5: Age Stratified Antioxidant Enzymes in Uterus Tissues

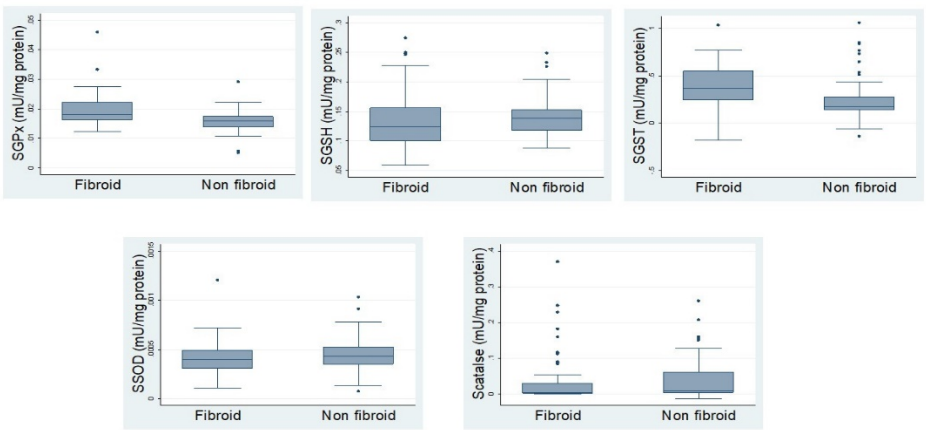
Groups		TGPx (mU/mg protein)	TGST (mU/mg protein)	TGSH (mU/mg protein)	TSOD (mU/mg protein)	TCAT (mU/mg protein)
B (26-35)	Case	0.0105±0.00	0.5967±0.05	0.0347±0.00	0.0002±0.00	0.029±0.01
	Control	0.0178±0.00	0.4173±0.04	0.0621±0.01	0.0002±0.00	0.015±0.01
	P-value	5.4 X 10^{-10}*	0.002*	0.005*	0.808	0.150
C (36-45)	Case	0.0126±0.00	0.2680±0.03	0.0285±0.00	0.0002±0.00	0.007±0.00
	Control	0.0138±0.01	0.0420±0.02	0.0646±0.01	0.003±0.00	0.015±0.01
	P-value	0.085	1.8 X 10^{-7}*	1.6 X 10^{-7}*	0.155	0.270
D (46-55)	Case	0.0159±0.00	0.226±0.05	0.0396±0.00	0.0003±0.00	0.014±0.00
	Control	0.0184±0.00	0.338±0.06	0.0466±0.01	0.0002±0.00	0.010±0.00
	P-value	0.051	0.169	0.570	0.218	0.731
E (56 & above)	Case	0.0183±0.00	0.2007±0.07	0.032±00.01	0.0002±0.00	0.005±0.00
	Control	0.0203±0.00	0.2857±0.04	0.1115±0.04	0.0002±0.00	0.012±0.01
	P-value	0.436	0.551	2.9 X 10^{-4}*	0.928	0.763

Values are expressed as mean ± Standard error of the means (SEM).

The asterisk*: signifies a significant difference.

TGPx: glutathione in tissue, TGST: glutathione-s-transferase in tissue, TGSH: reduced glutathione in tissue, TSOD: superoxide dismutase in tissue and TCAT: catalase in tissue

and non-UF participants. **Keys** - SGPx: glutathione peroxidase (in serum), SGST: glutathione-S-transferase (serum), SGSH: reduced glutathione (serum), SCAT: catalase and SSOD: superoxide dismutase (serum).



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3.1.6:. Concentrations of the Inflammatory Markers

Table 7a shows the concentrations distribution of inflammatory markers in UF and non-UF, while Table 7b shows the levels of the inflammatory markers in case-control stratified by age design. There was a significant ($P < 0.05$) increase in IL-2 concentration of UF patients compared to the non-UF participants. The mean concentration levels of IL-2 significantly increased at ($P < 0.05$) in group C in UF compared to the non-UF participants.

Table 6: Antioxidants in UF and Age-matched Non-UF in Serum

Groups		SGPx (mU/mg protein)	SGST (mU/mg protein)	SGSH (mU/mg protein)	SSOD (mU/mg protein)	SCAT (mU/mg protein)
B (26-35)	Case	0.00157±0.000	0.1413±0.014	0.1408±0.010	0.0005±0.000	0.0778±0.016
	Control	0.0230±0.001	0.2977±0.045	0.1334±0.006	0.0004±0.000	0.0683±0.010
	P-value	4.9×10^{-9}*	0.004*	0.477	0.061	0.486
C (36-45)	Case	0.0148±0.001	0.3922±0.036	0.1115±0.006	0.0004±0.000	0.0149±0.008
	Control	0.0188±0.001	0.2945±0.058	0.1415±0.005	0.0004±0.000	0.0044±0.001
	P-value	0.004*	0.065	0.026	0.054	0.444
D (46-55)	Case	0.0162±0.001	0.5091±0.044	0.1706±0.016	0.0004±0.000	0.007±0.003
	Control	0.0166±0.002	0.4935±0.064	0.1641±0.009	0.0004±0.000	0.0040±0.001
	P-value	0.797	0.834	0.673	0.722	0.863
E (56 & abv)	Case	0.0050±0.004	0.4850±0.031	0.1903±0.042	0.0004±0.000	0.0017±0.000
	Control	0.0197±0.001	0.3880±0.005	0.1410±0.00	0.0007±0.000	0.0540±0.001
	P-value	8.7×10^{-6}*	0.505	0.092	0.009*	0.183

The values were represented as mean ± standard error of the mean (SEM).

The asterisk*: signifies a significant difference.

SGPx: glutathione in serum, SGST: glutathione-s-transferase in serum, SGSH: reduced glutathione in serum, SSOD: superoxide dismutase in serum and SCAT: catalase in serum

Table 7a: Concentration of Inflammatory Markers

S/N	Inflammatory Markers (pg/mL)	Groups		
		Case	Control	Sig (2-tailed)
		Mean ±SEM		
1	IL-1	28.743± 8.568	13.700± 2.604	0.097
2	IL-2	197.885± 89.278	12.240± 5.862	0.041*
3	CoX-1	1495.443± 38.2294	1162.630 ± 47.386	0.390
4	CoX-2	2531.477± 82.523	2746.438± 97.878	0.867
5	TNF-α	113.753 ± 43.225	90.167± 33.079	0.666

Values = mean concentration and ± the standard error of the mean (SEM) of the inflammatory markers in myomas patients and non-myomas participants.

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*: signify the significant difference.
IL-1: Interleukin-1, IL-2: Interleukin-2, CoX-1: Cyclooxygenase-1, CoX-2: Cyclooxygenase-2 and TNF-α: Tumour necrosis alpha.

Table 4.7b: Inflammatory Markers in UF and Non-UF Participants.

Groups		IL-1 (Pg/mL)	IL-2 (Pg/mL)	CoX-1 (Pg/mL)	CoX-2 (Pg/mL)	TNF-α (Pg/mL)
B (26-35)	Case	8.78±0.52	35.50±15.70	2704.47±12.61	136080±73.12	47.53±6.55
	Control	8.01± 0.38	41.07±20.60	1913.56±11.22	439.33±22.79	34.18±.15
	P-values	0.963	0.973	0.496	0.690	0.895
C (36-45)	Case	43.82±19.26	389.59±19.78	1385.22±64.60	3475.53±164.95	197.45±88.73
	Control	18.02±5.66	197.47±12.34	741.89±15.27	5352.60±199.36	153.96±66.88
	P-value	0.058	0.004*	0.701	0.308	0.575
D (46-55)	Case	28.10± 5.69	66.15±28.87	975.13±14.77	2480.15±113.05	21.24±1.96
	Control	13.44±2.75	13.46±1.20	599.20±37.16	316.00±11.76	7.10±6.60
	P-value	0.393	0.722	0.806	0.438	0.907
E (56 & above	Case	7.61±0.32	0.00±0.00	676.06 ± 2.41	414.00±18.48	38.19±15.32
	Control	7.43±0.10	0.00±00	367.73 ±18.52	256. 04±6.24	36.27±10.66
	P-value	0.996	1.000	0.869	0.934	0.993

Values are expressed as mean ± Standard error of the means (SEM)

*: signifies significant differences.

IL-1α: interleukin-1 alpha, IL-2: interleukin-2, CoX-1: cyclooxygenase-1, CoX-2: cyclooxygenase -2 and TNF-α: tumour necrosis factor-alpha.

4. Discussion

4.1. Socio-demographic Characteristics of Participants

The obtained socio-demographic features of the participants via the questionnaire revealed an exciting result. The ethnicity assessment showed that the percentage of Yoruba that participated in the study surpassed other tribes. The result obtained in this study on the socio-demographic features of the participants agrees with previous studies [30]. The respondents residency is a function of the study location and the tertiary nature of the hospital.

4.2. Association of Micronutrients-vitamins on UF Growth

The results obtained were diverse between the case-control design and the age-stratified models. There were generally low serum concentrations of all the vitamins examined in the case compared to their respective control (Figure 1). The reduction was particularly more pronounced in the vitamin A level in UF cases in this study. The finding in this study contradicts the report of Martin, Huber, Thompson and Racine [11], where a high vitamin A level was assumed to support UF growth. However, Makwe, *et al.* [31] found no association in the level of vitamin A with UF growth. The reduction in the mean serum level

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of vitamin A in UF cases in this study may signify a decline in its antioxidant, antineoplastic, and immunity prowess. Studies have shown that low levels of vitamin A and certain micronutrients may play a part in UF development due to a decrease in their antioxidant and antineoplastic property [32].

The analysis of data on vitamin D in this study shows a pronounced reduction in vitamin D levels in UF patients (Figure 1). The lower vitamin D level associated with uterine fibroids in this study agrees with an earlier report by Makwe, Soibi-Harry, Rimi, Ugwu, Ajayi, Adesina, Okunade, Oluwole and Anorlu [31]. Low vitamin D level was implicated in the growth of many gynaecological diseases [20,33]. The decrease in vitamin D levels among myoma patients was peculiar to symptomatic uterine fibroid women. The conclusion derived was that patients with a big UF volume had lower vitamin D levels in their blood [34]. An *in-vivo* study also revealed that the size of UF also reduced with vitamin D treatment [32]. Thus, this study agrees with several studies that have recognised vitamin D insufficiency or deficiency as a uterine fibroids risk factor [31].

This study reveals a conspicuous reduction in the level of vitamin E in UF women. This finding contradicts the findings of Martin, Huber, Thompson and Racine [11] and Wise, Radin, Palmer, Kumanyika, Boggs and Rosenberg [9] that reported no correlation between vitamin E and UF development. However, Ciebiera, Esfandyari, Siblini, Prince, Elkafas, Wojtyla, Al-Hendy and Ali [18] demonstrated an association of UF with a high level of α- tocopherol (vitamin E vitamer) that influences genetic polymorphisms. The low vitamin E recorded amounts to a decrease in antioxidant activity and the establishment of oxidative stress, which is peculiar to UF compared to normal myometrium. According to Fletcher, *et al.* [35], high oxidative stress was related to decrease apoptosis which could influence the transformation of the healthy myometrial cell into fibroid-like cells [20,35].

The vitamin K level in UF cases was remarkably higher in this study. However, studies on the correlation between UF and vitamin K are sparse, limiting the opportunity to compare this result with previous findings. Vitamin K plays an appreciable role in coagulation, anti-inflammatory, and anticarcinogen in cancer models. Vitamin K's anti-inflammatory and anti-cancer properties prevent cancer growth that shares similar risk factors with UF [20]. A high level of vitamin K in this study may be due to rupture of the red blood cells leading to an anaemic condition that can influence inflammation. The stress involved in the development and sustenance of UF can induce rupture of the red blood cell following the pattern reported in other diseases [36]. The remarkable influence of UF growth on these vitamins could be related to differences in age. Age advancement may cause declination in physiological functions that can be a prerequisite to nutritional inadequacy or imbalance [31,37].

4.3. Micro-elements and UF Progression

The mineral elements analysis outcome shows that the mineral elements are associated with UF growth. Although the case-control design model only showed selenium was significantly decreased in UF patients compared to the non-UF participants. Low concentration levels of selenium could be attributed to the progression of UF, and the administration of selenium supplements strongly decreased UF diameter in Japanese quail [38]. The association of selenium supplementation in reducing cancer progression was earlier reported [39]. The present results showed a remarkable reduction in selenium concentration among the UF patients compared to the control.

The determination of micronutrient elements in UF and non-UF participants in the age-stratified design model showed age's influence on the microelement markers. The inconsistency in the outcome of each mineral element may be due to the influence of age and the nutritional imbalance of each individual in the study population. The increase in Na and Fe concentration with a decrease in Zn concentration in UF patients agrees with the previous report [40]. Selenium concentration level was lower in group D. Investigating selenium in human uterine fibroid has not been documented to the best of our knowledge. Although, a recent research study on selenium shows that selenium supplements reduced

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leiomyosarcomas and other tumour types in Japanese animals [38]. Other mineral elements (magnesium, calcium, potassium) have varied concentration levels in different age groups. Thus, the diversity in each mineral element concentrations in different age groups may be related to individuals' age and environmental factors in the study populace (Table 4).

High level of sodium salts that is above WHO recommendation was associated with diets in Africa and even among Blacks in America [41-44]. The implication was restricted to the high incidence of hypertension and cardiovascular disease [45]. This study implicates the high sodium salt diets to the high level of sodium in the uterus of fibroid patients, and therefore a main cause of the disease along with vitamin D deficiency among black people. To corroborate the findings in this study, we proposed the high sodium content in sodium monoglutamate that has been implicated in the development of fibroid in women [46]. High potassium in diets reverses high blood pressure due to high sodium salt intake [47]. This same strategy is a possible solution to prevent, alleviate or reverse fibroid in women. A greater dimension of understanding of leakage or accumulation of sodium ions is required for exploitation into the control of fibroid in women. The structure of the appropriate channels for sodium was recently elucidated in this direction and for a possible drug target [48]. A study implicating some micronutrients and UF in rats showed that the abundance of these minerals in fibroids tissue follows this order $Na^{+}>K^{+}>Ca^{2+}>Mg^{2+}>Fe^{2+}>Zn^{2+}$. Although, they could not associate their findings with the initiation or proliferation of UF tissues [49]. Nutrition has been proved to have imprinting effects on the human genome during the last decade. Many research indicates that early life nutrition may influence the chance of developing chronic diseases later in life [50]. Although there was a lack of information on the correlation of sodium with uterine fibroid, one study revealed that sodium concentration level was greater than other micronutrients in intramural uterine fibroid [49].

Frequent hydration in the tropical heat is a possible cause of the high preference for sodium salt intake in Africa and other tropical countries. The sodium intake in the diet recommended is between 2.5–6.0 g/day (6.4–15.3 g/day salt) or 50-250 mmol/day for optimal health [51-53]. Natriuretic hormones enhance increased excretion of intracellular sodium [54]. Thus, there is a high possibility of their use in the clinical management or the prevention of fibroid. The susceptibility of women to the development of fibroids may vary due to diversity in preferences for sodium intake [55]. This susceptibility may be more pronounced in women with an inherited disorder of renal salt homeostasis [56]. Frequent water intake in women, which facilitates the excreation of sodium, is a possible strategy to prevent fibroid development. The application of pressure natriuresis, or sustained renal sympathoinhibition to treat or prevent fibroid from encouraging high excretion of urinary sodium salts, should be exploited [51,57,58].

The growth and development of UF are strongly associated with the nutritional influence of vitamins A ($P= 0.006$), vitamin D ($P= 0.006$), vitamin E (< 0.001), vitamin K ($P= 0.019$) and selenium ($P =0.046$). Vitamin D does not work alone. The intake of vitamin D in the absence of sufficient mineral elements results in the influx of toxic xenobiotics and some negative clinical outcomes. Achieving a suitable balance between minerals and vitamin D in biological systems to avoid harm and illness was recommended [4]. Vitamin D_3 influences the Na^{+}-K^{+}-ATPase, other transporter systems and the homeostatic balance of minerals like zinc, manganese and iron [59].

4.4. Antioxidants Activities

4.4.1. Antioxidant Status in Tissue of Myomas Patients

The activity of the antioxidant enzymes in tissue homogenate of UF patients and non-UF participants yielded diverse outcomes. The concentration values of GPx and GSH were reduced in the tumour patients, in contrast to the high GST concentration level in UF patients. The remarkable change noticed in the UF women in GPx and GSH was consistent

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with earlier report [60,61]. Thus, the concentration levels of the antioxidant enzymes in UF women in four of the five examined antioxidant markers were low (Figure 3). This result was consistent with earlier report [62]. Studies have established that uterine fibroids have impaired antioxidant activity, with oxidative stress being a distinct player in most gynaecological issues [35]. The oxidative stress could be why the decrease in most of the antioxidant activities in the UF women in this present study.

In the age-stratified design model for antioxidants activity, Gpx and GSH were conspicuously reduced in the case compared to the age-matched control in group B (26-35). This corroborates with other studies [60,61]. However, there was a significant increase in the mean concentration level of GST in groups B and C. In contrast, GSH was reduced remarkably in the case of groups C and E. The inconsistencies observed in the antioxidant and elemental results might be due to age, lifestyle and adjuvant taken in advanced age by the elderly [63].

4.4.2. Assessment of the Antioxidant Status in Serum

The antioxidant enzyme activities in blood serum showed different patterns (Figure 4). The GPx and GST were increased in the UF compared with the non-UF. The other three antioxidant enzymes (GSH, SOD and CAT) were insignificant. The high values obtained for GPx and GST are inconsistent with earlier report [63]. Thus, in the neoplastic tumour, activities of the antioxidant enzymes follow different patterns, especially for uterine fibroids tumours with multiple risk factors. The age-stratified design model in serum antioxidants shows a significant reduction in mean concentration levels of GPx, GST, and SOD in groups B, C and E, respectively. The result obtained agrees with other studies [60,61]. The decrease in the concentration levels of these antioxidant enzymes could result from the impairment of these antioxidant enzymes

4.5. Inflammatory Cytokines in UF

The outcome of inflammatory cytokines showed that IL-2 is correlated to UF growth and development in both case-control and case-control stratified by age models. There was a significant increase in the concentration level of IL-2 in UF patients compared to the control. However, the case-control stratified model saw a remarkable increase in IL-2 concentrated level in group C (Table 7a). The result agrees with the report [18,64]. The increase in cytokine concentration level could result from a microenvironment created by local chronic inflammation, which promotes the development of UFs.

Myomas development has been linked to numerous inflammatory-derived chemicals in the myometrium [18]. Changes in the levels of the inflammatory cytokines could also result from the dietary factors that contribute to the aetiology and growth of UF. Research studies have shown that dietary trans-fat produces inflammatory indicators and pro-inflammatory cytokines that influence the secretion of enzymes in the endometrial extracellular matrix. Thus, diet can manipulate and modify inflammation and endogenous hormone [8,18,65]. Analysis of inflammatory markers based on age grouping suggests an increase in IL-2 in the UF patients of group C (Table 2). This finding is similar to the report from other studies [18,64].

In summary, the development of UF may typically follow concerted actions of vitamin A deficiency, high sodium salt intake, and the influence of interleukin-2. IL-2 regulates sodium-current flux [66,67]. Therefore, the induced proliferation of UF could be due to the overwhelming influence of excess sodium ions obtained through diets on IL-2. A mutant IL-2 was implicated in inhibiting the progression of a similar tumour, which gives credence to the postulated role of IL-2 in the growth of UF in this study [68]. The influence of the interaction of IL-2 with either zinc, vitamins B1, D, or particularly vitamin A that was implicated in the propagation of UF in this study was reported [69-73].

5. Conclusions

Thus, prolonged vitamin A deficiency coupled with excess sodium salt are critical factors for UF development. A critical stage of UF development may be facilitated by inflammation induced by IL-2 and CoX-1, coupled with long-time cumulative action of excess intake of the myriad of micronutrients between 36-45 years among women. A concurrent increase in the activity of glutathione-s-transferase and the reduction in glutathione peroxidase activity in the uterus may be a biomarker for early detection of UF development.

Supplementary Materials: The following are available online, Appendix S1: Informed consent form designed and approved for the study, Appendix S2: Participants questionnaire form designed and approved for the study.

Author Contributions: Conceptualization, M.A.O, O.O.O. and I.S.A.; Formal analysis, M.A.O, O.O.O. and I.S.A.; Investigation, M.A.O, F.A.B., O.O.O. and I.S.A.; Methodology, M.A.O, F.A.B., O.O.O. and I.S.A.; Data curation, M.A.O, O.O.O. and I.S.A.; Project administration, M.A.O, F.A.B., O.O.O. and I.S.A.; Supervision, F.A.B., O.O.O and I.S.A.; Visualization, M.A.O, O.O.O. and I.S.A.; Writing – original draft, M.A.O, O.O.O. and I.S.A.; Writing – review & editing, M.A.O, F.A.B., O.O.O. and I.S.A.

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Institutional Review Board Statement: This study involved human subjects. "The research described in the submitted protocol and other related documents has undergone a positive review and given a full approval (protocol code, CHREC/38/2020; date of approval, 23rd January 2020) following the outcome of the review by the Covenant Health Research Ethics Committee (CHREC) of Covenant University, Ota, Nigeria. There was no adverse event experienced to report to CHREC during the study" The study was conducted according to the guidelines of the CHREC, operating under the National Code for Health Research Ethics (NHREC/25/10/2018), and the US Department of Health & Human Services (IORG0010037). The protocol was subjected to additional review, and full approval (protocol code, UI/EC/19/0268; date of approval, 19th September 2019) was obtained from the UI/UCH Ethics Committee of the Institute for Advanced Medical Research and Training (IAMRT) of the College of Medicine, University of Ibadan, Ibadan, Nigeria operating under the National Code for Health Research Ethics (NHREC/05/01/2005a).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study. All informed consent was documented in writing.

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