Insights into Cardiovascular Risk and Nutritional Status in Subjects with Wheat-related Disorders

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Running title: Cardiovascular Risk and Nutritional status in Wheat-related Disorders

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ABSTRACT

Objective: Wheat-related disorders are a spectrum of disorders associated with different autoimmune and non-autoimmune diseases. However, it is unclear whether these wheat-related disorders lead to adverse health effects such as cardiovascular risk, nutritional deficiencies etc. The objective of the study was to explore the lipid profiles and the nutritional status of subjects with wheat-related disorders to understand the potential threat by wheat on cardiovascular risk and nutritional deficiency.

Method: A total of 1040 subjects who showed wheat-related symptoms were initially tested for the wheat protein antibody panel (Wheat Zoomer panel and Celiac Disease panel), then for cardiovascular panel and the micronutrient panel at Vibrant America Clinical Laboratory.

Results: Subjects with both Wheat Zoomer positivity (WZ+) and celiac disease positivity had significantly low levels of high-density lipoproteins (HDL) (279/483(57.8%) and 29/47(61.7%) respectively), but only subjects with WZ+ had low levels of apo A1 (44/424(9.5%)), and high levels of Omega 6 fatty acids (53/334(15.9%)). None of the micronutrients tested showed a significant imbalance in WZ+ subjects.

Conclusion: Subjects with positive serology for Wheat Zoomer have deranged blood lipid profiles but did not show any significant micronutrient deficiency. Hence, our results showcase a significant association of wheat-related disorders to cardiovascular risk.

KEY WORDS: Wheat sensitivity, Celiac disease, Lipid Profile, Micro nutrients, Cardiovascular Risk

Clinical Significance:

- The study was focused on analyzing the association of cardiovascular and nutritional risk in subjects from a wide spectrum of wheat-related disorders.
- The positive serology for wheat related disorders was based on a comprehensive set of 18 key wheat protein antibody panel (wheat Zoomer (WZ) and Celiac Disease Panel (CD)).
- Subjects with positive serology for Wheat Zoomer Showed deranged blood lipid profiles with low HDL, low apo A1 and high levels of Omega 6.
- Positive serology for wheat zoomer did not affect the micro nutrient status.

INTRODUCTION

Wheat is considered one of the most extensively grown and consumed grain in the world.[1] The major structural compound in wheat is gluten which is also considered as the culprit for many wheat related disorders. Some individuals have adverse effects after ingesting gluten, specially the toxic protein portion of gluten which include gliadin and glutenin. These individuals may experience gastrointestinal symptoms, such as abdominal cramps, diarrhea, gas, nausea vomiting, bloating and extra intestinal symptoms such as fatigue, joint pain, depression, and cognitive difficulties. Wheat-related disorders are not one disease but a spectrum of diseases, including celiac disease (CD) and wheat allergy (WA) which are the best known to date.[2] Celiac disease is an inflammatory autoimmune disease triggered by gluten in genetically predisposed individuals. It is characterized by specific serological markers and small intestinal damage with loss of absorptive villi, typically leading to malabsorption of nutrients.[3] The prevalence of CD is estimated to be 0.5-1.0%. In contrast, WA is not considered an autoimmune disease, but an IgE-mediated reaction to the gliadin portion in gluten. WA is considered one of the top 8 allergies in the United states with a 0.1% documented prevalence in westernized countries.[4] Besides CD and WA, some individuals suffer from both gastrointestinal and extra-gastrointestinal symptoms caused by wheat in the absence of CD and WA. This is an emerging entity in the spectrum of wheat related disorders known as non-celiac-wheat-sensitivity (NCWS). The prevalence of NCWS and the demographic characteristics are relatively lacking[5], but some studies report it to be 0.55–6 % in the USA population.[6-8] Currently, no reliable biomarker is available for accurate diagnosis of NCSW, hence confirmatory tests are used after CD and WA have been appropriately ruled out.[5, 9]

Wheat-related disorders are known to be associated with many other diseases. The association between celiac disease and other autoimmune diseases such as autoimmune thyroid disease, Addison's disease, autoimmune insulin-dependent diabetes mellitus, Sjogren's syndrome etc. are widely studied. But, information on the association of wheat-related disorders with non-autoimmune diseases are relatively limited. The cardiovascular risk is one of many since the deranged blood lipid profiles, abnormalities in adiposity, and other risk factors for cardiovascular disease in CD patients are not clearly presented. West et al. showed that subjects with CD on gluten-free-diet (GFD) has lower prevalence of hypertension, which is another cardiovascular disease marker, than in the general population hence could be a protective factor, but many other studies suggest CD to be a risk factor for cardiovascular disease.[10-11] Unfortunately, these studies were not structured appropriately to assess these two factors (CD without GFD Vs. CD with GFD) separately hence it is not clear whether the protective or risk factor is associated with CD itself or the use of GFD.[12-14] Individuals with CD have also been shown to have nutritional deficiencies as a result of malabsorption in the damaged villi. Unfortunately, most studies have only concerned on the association of CD with these disorders but not on other entities in the spectrum of wheat-related disorders.

In this study, we sought to asses the blood lipid profiles of individuals with seropositive celiac disease panel and wheat protein antibody panel known as Wheat Zoomer to understand any derangements that could

lead to cardiovascular risk and nutrient deficiency. We tested IgG/IgA to 16 key wheat protein antigens including celiac disease markers, and simultaneously tested the same subjects for cardiovascular panel including lipids, Apolipoproteins, Inflammation markers, Myocardial stress markers, lipoprotein markers and fatty acids and micronutrient panel including 72 different intracellular and extracellular vitamins, minerals, antioxidants, fatty acids, amino acids and electrolytes. Our results clearly showed that subjects who were seropositive for wheat protein antibodies without any positivity in CD panel had significantly low levels of HDL, and apo A1 and high levels of Omega 6 fatty acids, which are risk factors in cardiovascular disease but not significant nutrient deficiencies.

MATERIAL AND METHODS

Patient Selection and Study Design

A total of 1040 subjects who showed symptoms after wheat ingestion were tested at Vibrant America Clinical Laboratory initially for the Celiac disease panel (CD), Wheat zoomer panel (WZ), and then for cardiovascular panel and micronutrient panel between October 2015 to June 2018. No restricted diet was instructed to the subjects during the study period.

For analysis purposes, subjects were defined into three groups. (Figure 1, flow chart)

- Celiac disease subjects (CD+) – Subjects who were seropositive for at least one antibody in the celiac disease panel despite the presence/absence of antibodies in the Wheat Zoomer panel. These subjects are celiac disease suspects only based on symptoms and serology tests (sensitivity 99%, specificity 100%)
- Wheat Zoomer positive subjects (WZ+) – Subjects who were seropositive for at least one antibody in the Wheat Zoomer panel but seronegative for any antibody in the celiac disease panel.
- Seronegative control subjects (NEG) – Subjects who were seronegative for any antibody in both Wheat Zoomer panel and celiac disease panel.
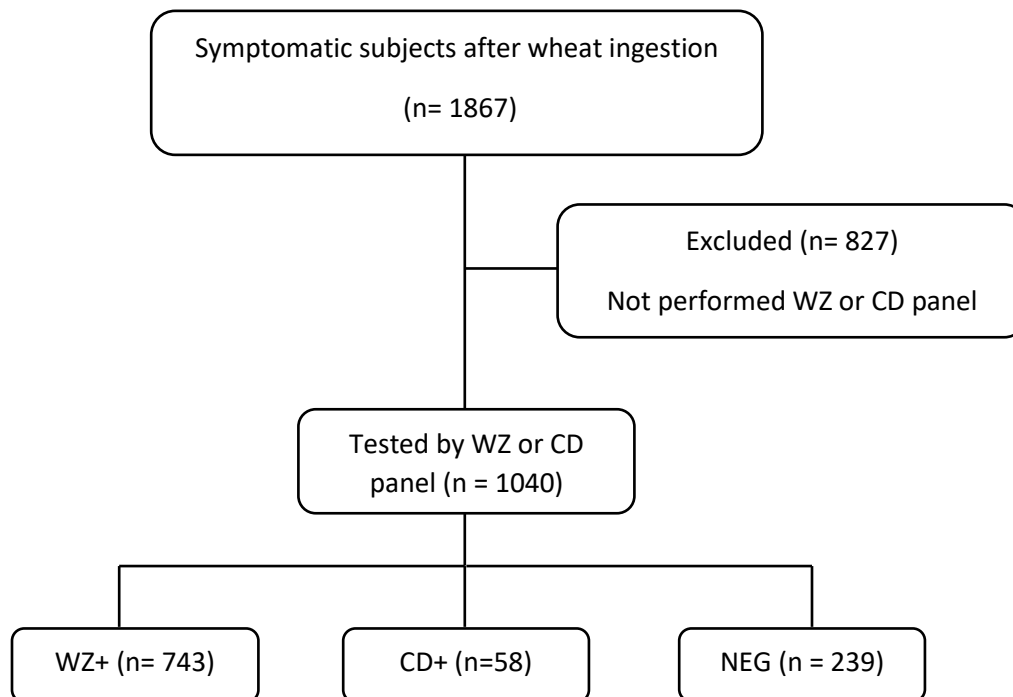
Figure 1. Flow diagram of the study population

Celiac Disease Panel

The celiac disease panel (Vibrant America, LLC, San Carlos, CA, USA) detects anti-tissue transglutaminase 2 (tTG) IgA and IgG, anti-deamidated gliadin peptide (DGP) IgA and IgG. A combined serologic test to detect these two antibodies on the fluorescence microarray platform was used for all Celiac disease diagnosis. The fabrication and validation of the arrays were very similar to the manufacture procedures described in our previous work.[15] In brief, an immunoassay was performed on the microarray and then the bound peptide-antibody complex was scanned via a fluorescence microarray scanner to generate the intensity of binding referred to the mean signal binding intensity of the subsequences. The results were interpreted by comparison with calibrators, controls and cut-off values. The sample was considered negative if the concentration of the antibody was equal to or less than the cut-off value chosen.

Wheat Zoomer

Wheat Zoomer (Vibrant America, LLC, San Carlos, CA, USA) is a comprehensive wheat protein antibody panel that detects IgG and IgA to 16 key wheat proteins (Transglutaminase 3, Transglutaminase 6, Wheat Germ Agglutinin, α Gliadin, α-β Gliadin, γ-Gliadin, Ω Gliadin, Gluteomorphin, Prodynorphin, Low molecular weight glutenin, High molecular weight glutenin, Serpin, Farnins, Amylase/Protease Inhibitors, Globulins, Purinin). It uses a peptide microarray containing a wide range of wheat-derived peptides. The method for generating the peptide array and testing protocol was similar to our previous work.[15] All the key proteins of wheat are arrayed on the Vibrant Wheat Zoomer chip as overlapping 18-mer peptides covering the entire

protein. These chips are then placed on a 96-pillar plate and assayed against samples to determine their reactivity.

Cardiovascular Panel

The cardiovascular panel comprised of lipids (total cholesterol, HDL, LDL, triglycerides), apolipoproteins (apo A1, Apo B), Inflammation markers (Lp PLA2, Homocysteine), Myocardial stress marker (NT-proBNP), lipoprotein markers (sdLDL, Lp(a)) and fatty acids (Omega-3, Omega-6). The cholesterols (HDL, LDL, total cholesterol) and triglycerides were assessed using a chromatographic enzymatic method. An immunoturbidimetric assay was used to measure Apo A1, apo B, and Lp(a) separately. The lipoprotein marker sdLDL was measured by reacting with multiple well-characterized surfactants and enzymes and analyzed via the Roche cobas 6000 c501 analyzer. Lp PLA2 and homocysteine were enzymatic assays measured indirectly via spectrophotometry. The Omega-3 and Omega-6 fatty acids assay involved measuring the percentage composition of the fatty acids in RBC phospholipids. Phospholipids were isolated from lyzed RBCs to generate free fatty acids by potassium hydroxide. The fatty acids generated are resolved and measured according to their hydrophobicity using LC-MS on the Xevo-TQD mass-spectrometer.

Micronutrient Panel

The LC-MS on the Xevo-TQD mass-spectrometer was used to measure the levels of fat soluble vitamins in WBC, fat soluble vitamins in plasma and serum, folate in red blood cells, water soluble vitamins in WBC and water-soluble vitamins in serum. ICP-MS was used to measure Mg, Fe in RBC, Cu, Zn, Se, Cr, Mn in serum, Ca, Cu, Zn, Se, Cr, Mn in WBC.

Patient and Public Involvement

This study does not include any patient or public involvement and the study is based on retrospective analysis of de-identified laboratory data, hence was exempted from formal ethical reviews by Western Institutional Review Board (WIRB).

Statistical Analysis

The retrospective analysis on clinical data from de-identified subjects were performed via Java for Windows version 1.8.161 and R for Windows version 3.5.0. Data were expressed as mean ± standard deviation (SD) when the distribution was Gaussian. Pearson's Chi-squared Test was used when the observed count is < 5 to evaluate the association between the presence of clinical variables evaluated. P value < 0.05 was considered statistically significant.

RESULTS

Prevalence of cardiovascular markers in subjects with positive Wheat Zoomer serology

The retrospective study was completed using deidentified clinical data and test results. After excluding incomplete clinical data, a total of 1040 subjects who showed symptoms related to wheat ingestion were tested initially for celiac disease panel, Wheat Zoomer panel, and then for cardiovascular panel. Table 1 shows the demographics and a detailed prevalence of each cardiovascular marker in Wheat Zoomer positive subjects, celiac positive subjects, and negative controls.

Table 1. Demographics and Prevalence of Cardiovascular markers in Celiac positive, Wheat Zoomer positive and Wheat negative subjects.

Cardiovascular Marker	CD+	WZ+	NEG
Age (X±SD)	45±17	47±16	49±17
Sex	35 F / 18 M	505 F / 221 M	163 F / 76 M
Lipid Profile			
High-density lipoproteins (HDL)	29/47(61.7%)	368/650(56.6%)	89/213(41.8%)
Low-density lipoproteins (LDL)	27/43(62.8%)	421/586 (71.8%)	142/194(73.2%)
Triglycerides	5/45(11.1%)	109/638(17.1%)	29/206(14.1)
Total Cholesterol	16/45(35.6%)	276/638(43.3%)	98/206(47.6%)
Apolipoproteins			
Apo A-1	5/42(11.9%)	53/586(9.0%)	8/200(4.0%)
Apo B	20/42(47.6%)	282/583(48.4%)	101/193(52.3%)
Fatty Acids			
Alpha-linolenic acid (ALA)	9/38(23.7%)	104/394(26.4%)	35/139(25.2%)
Eicosapentaenoic acid (EPA)	21/38(55.3%)	158/394(40.1%)	66/139(47.5%)
Docosapentaenoic acid (DPA)	14/38(36.8%)	251/394(63.7%)	75/139(54.0%)
Docosahexaenoic acid (DHA)	11/38(28.9%)	113/394(28.7%)	37/139(26.6%)
Linoleic acid (LA)	9/38(23.7%)	168/394(42.6%)	47/139(33.8%)
Arachidonic acid (AA)	14/38(36.8%)	158/394(40.1%)	62/139(44.6%)
Omega 3	8/38(21.1%)	78/394(20.4%)	32/139(23.0%)
Omega-3 Index	28/38(34.2%)	319/394(90.0%)	105/139(75.5%)
Omega 6	3/38(7.9%)	58/394(14.7%)	10/139(7.2%)
Inflammation			
Lp-PLA2[a]	7/43(16.3%)	105/607 (17.3%)	39/199(19.6%)
Homocysteine	24/46(52.2%)	273/658(41.5%)	100/210(47.6%)
Myocardial Stress			
NT-proBNP[b]	3/40(7.5%)	20/562(3.6%)	10/181(5.5%)
Lipoproteins			
Small-density LDL (sdLDL)	4/44(9.1%)	164/575(28.5%)	98/190(25.3%)
Lipoprotein(a) (Lp(a))	6/45(13.35)	174/590(29.5%)	57/192(29.7%)

| Coenzyme Q10(CoQ10) | 26/34(76.5%) | 328/418(78.5%) | 88/104(84.6%) |

aLipoprotein-associated phospholipase A2 (Lp-PLA2), bN-terminal pro-brain natriuretic peptide (NT-proBNP)

As shown in figure 2. both CD+ and WZ+ individuals showed low levels of HDL (p= 0.0153 and 0.0002) and WZ+ individuals showed low levels of APO A-1 (p= 0.0212) and high levels of Omega-6 (p= 0.0258) compared to negative controls.
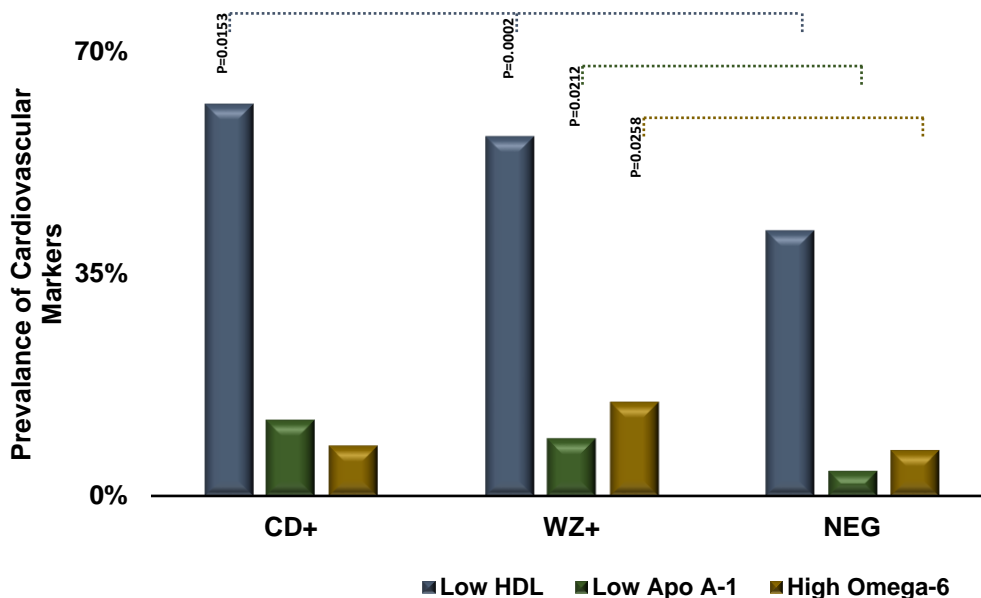

Figure 2. Prevalence of HDL, Apo A-1 and Omega-6 in celiac positive subjects (CD+), Wheat Zoomer positive subjects (WZ+) and negative controls (NEG). The entities without any p values (low apo A1 and high Omega 6 in CD+) did not show any significant difference compared to the negative control group.

Micronutrient status of individuals with positive Wheat Zoomer serology

Next, we sought to evaluate the relationship between micronutrient status with Wheat Zoomer serology status. We chose a subset of participants from the initial study population who had Wheat Zoomer positive serology and tested for Micronutrient levels. Table SI1 shows the status of the 72 biomarkers for nutritional status (34 extracellular markers and 38 intracellular markers) in Wheat Zoomer positive serology subjects compared to negative controls. None of the nutritional markers showed a significant deficiency or elevation in subjects with positive Wheat Zoomer serology compared to negative controls.

DISCUSSION

The association of wheat-related disorders with other autoimmune and non-autoimmune diseases are studied widely but unfortunately, most of the studies have concentered on CD, since it is the best known to date. CD is only one entity in the spectrum of wheat-related disorders hence our objective of this study was to evaluate the association between subjects with positive wheat-related serology (including CD) and biomarkers of cardiovascular disease and nutritional status. The grouping of the subjects was based on the CD protein antibodies and other wheat protein antibodies. Wheat Zoomer has peptides for 16 different key proteins that are known to play a vital role in wheat-related disorders, hence covers a wide array of protein antigens that can cause wheat-related problems. In our study, the WZ+ subjects (without positive CD serology) were given high priority hence none of the studies have focused on this entity to find the association with cardiovascular risk and risk for nutritional deficiencies.

As illustrated in figure 2, we observed significant differences in lipid profiles in subjects with positive Wheat Zoomer serology compared to those with negative serology both for Wheat Zoomer and celiac. We specifically found that both Wheat Zoomer positive subjects and CD positive subjects had significantly lower levels of HDL-cholesterol, but only Wheat Zoomer positive subjects had significantly lower apo A1, and higher Omega-6 levels compared to the controls (seronegative for both celiac and Wheat Zoomer).

Apo A1 is a lipoprotein produced and secreted in the intestinal mucosa and one of the main lipoprotein constituents in HDL cholesterol. The inflammation caused in the intestinal mucosa due to wheat ingestion could disrupt and reduce the apo A1 production and secretion thus in fact reducing the synthesis of HDL.[16] The malabsorption in the intestinal mucosa due to the possible damages to the villi in subjects with positive CD serology would explain the higher percentages of lower HDL individuals in CD+ (61.7%) compared to WZ+ (56.6%) and NEG (41.8%) subjects. Previous studies found that HDL and apo A1 were low in subjects with positive CD serology,[17] but none of the studies have reported any relationship with other wheat-related disorders. For the first time, our study provide evidence that WZ+ subjects, similar to previously reported CD+ subjects, showed low levels of HDL and apo A1. WZ+ and CD+ subjects are close entities in the spectrum of wheat-related disorders, hence could assume similar characteristics. Omega-6 is a known acute phase protein that could be elevated due to inflammation caused by the ingestion of wheat.[18] In support of this mechanism, we observed that Omega-6 was significantly elevated in subjects with WZ+ serology compared to the negative controls. Thus, similar to CD, we were able to provide evidence for the risk of developing unfavorable lipid profiles in subjects with wheat-related disorders other than celiac disease.

Another possible adverse health effect similar to deranged lipid profiles that CD patients often suffer from is the nutritional deficiencies. Several studies have reported CD patients to be nutrient deficient due to the malabsorption through the damaged villi.[19] Hence, we measured the nutritional status of the subjects with positive Wheat Zoomer serology. The micronutrients test was performed both in extracellular plasma and inside(intracellular) red blood cells (RBC) and white blood cells (WBC). The extracellular micronutrient levels provide a snapshot of the status of micronutrient baseline levels at a given time. It is a reflection of a

person's diet over a narrow period of time.[20] In contrast, the intracellular levels of micronutrients provide the information on the absorbed levels of the nutrients, thus accounts for the factors such as genetic, aging, lifestyle, chronic illness, medication etc. that could interfere the absorption and can change the functional nutritional levels.[21] However, our results did not show any significant difference in micronutrient levels in subjects with positive Wheat Zoomer serology compared to negative controls. This difference with other reported CD studies and our Wheat Zoomer results could be explained by the malabsorption due to possible damages in villi that is often seen in CD patients and but not in other wheat-related disorders. A confirmatory biopsy test may be required to prove this hypothesis since our study only includes subjects based on their serology testing.

CONCLUSION

We assessed various biomarkers related to cardiovascular health and nutritional health in subjects with positive serology for CD and WZ. Subjects with positive serology for WZ and CD showed unfavorable lipid profiles in terms of low HDL (both WZ+ and CD+), low apo A1(only WZ+) and high levels of Omega 6 (only WZ+) compared to the negative controls but did not show any significant changes in micronutrient levels. Our results demonstrate a robust association of wheat-related disorders to cardiovascular risk.

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DECLARATION OF CONFLICT OF INTEREST

Siriwardhane is an employee of Vibrant America LLC. Krishna, Devarajan, Ranganathan, Jayaraman, Wang, Bei, Rajasekaran, Krishnamurthy, are employees of Vibrant Sciences LLC.*, CA, USA*

COMPLIANCE WITH ETHICAL STANDARDS

This study is based on retrospective analysis of de-identified laboratory data, hence was exempted from formal ethical reviews by Western Institutional Review Board (WIRB). The data and materials in this manuscript have not been published elsewhere and are not under consideration by another journal.

AUTHOR CONTRIBUTIONS

TS, HK, KK, KD and TW performed the research. TS, HK, JJ, and VJ designed the study. TS, HK, KB and VR analyzed the data. TS and HK wrote the article.

AVAILABILITY OF DATA AND MATERIAL

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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