Published in final edited form as:

J Psychopharmacol. 2014 April ; 28(4): 349–356. doi:10.1177/0269881113516200.

Endothelial nitric oxide synthetase genetic variants, metabolic syndrome and endothelial function in schizophrenia

KJ Burghardt, PharmD¹, TB Grove, BS^{1,2}, and VE Ellingrod, PharmD^{1,3}

¹Department of Clinical Social and Administrative Sciences, College of Pharmacy. University of Michigan, 428 Church St., Ann Arbor, MI, 48109. United States

²Department of Psychology, School of Liberal Arts and Science, University of Michigan, 530 Church St., Ann Arbor, MI, 48109. United States

³Department of Psychiatry, School of Medicine, University of Michigan, 4250 Plymouth Rd., Ann Arbor, MI, 48109. United States

Abstract

Objective—The increasing rates of metabolic syndrome and cardiovascular disease in schizophrenia has led to investigation into their causes including atypical antipsychotics and pharmacogenetic variants. This study focuses on the peripheral vasculature as a cardiovascular phenotype and the influence of atypical antipsychotics, the aberrant metabolism of nitric oxide caused by endothelial nitric oxide synthetase (*eNOS*) genetic variants and metabolic syndrome in a cross-sectional sample of schizophrenia subjects.

Methods—Associations between *eNOS* genetic variants and endothelial function was assessed in a cohort of schizophrenia patients taking antipsychotics, undergoing a clinical assessment for endothelial function via the peripheral artery tonometry (RH-PAT) method as well as a metabolic syndrome criteria screening. Analyses were conducted on the entire cohort then again after stratifying by metabolic syndrome to investigate the effect of the *eNOS* variants and metabolic syndrome on endothelial functioning.

Results—203 subjects with a mean age of 46 years were included. The cohort was 36% female, 36% had metabolic syndrome and 85% were currently using atypical antipsychotics. Associations between the *eNOS* T⁻⁷⁸⁶C and worse endothelial functioning (lower RH-PAT values) were found only in schizophrenia patients *without* metabolic syndrome.

Conclusions—Our results suggest that when schizophrenia patients progress to meet metabolic syndrome criteria, the genetic protection of the *eNOS* T⁻⁷⁸⁶C variant on endothelial function is no longer seen and other factors of this pro-inflammatory state may be overriding this effect. The results of this study need replication and the factors driving endothelial dysfunction in patients with metabolic syndrome warrant further investigation.

Keywords

Schizophrenia; Endothelial; Antipsychotics; Pharmacogenetics; Nitric Oxide Synthetase

Corresponding Author: Vicki L. Ellingrod, PharmD, FCCP, vellingr@umich.edu.

Present Address: 428 Church Street Ann Arbor, Michigan 48109, Telephone #: 734-615-4728, Fax#: 734-763-4480

Introduction

The incidences of metabolic syndrome and cardiovascular disease in schizophrenia have dramatically increased since the advent of the more commonly prescribed atypical antipsychotics (AAPs) (Hennekens et al., 2005; Laursen et al., 2012; McEvoy et al., 2005). Explorations into the causes, predictors and interventions; with the goal of ameliorating these side effects in schizophrenia have been numerous and include diet, lifestyle and personalized medication interventions. However, to date, there has been little investigation looking at the peripheral vasculature as a cardiovascular phenotype in schizophrenia and the ways atypical antipsychotics, comorbid disease states, and genetics play a role in its functioning.

The metabolic syndrome is comprised of metabolic risk factors for type 2 diabetes and cardiovascular disease (CVD). These risk factors include abdominal obesity, insulin resistance, dyslipidemia and hypertension. Metabolic syndrome increases the risk of CVD and all-cause mortality in schizophrenia and is thought to be associated with a pro-inflammatory state (Ford, 2005; Galassi et al., 2006; Gami et al., 2007; Grundy et al., 2005; Koh et al., 2005; Prossin et al., 2013). The endothelium is an active biologic interface lining arteries and veins. It regulates growth, tone, hemostasis and inflammation in the circulatory system. Repeated insults to the vascular endothelium results in an inflammatory response and endothelial dysfunction. Dysfunction of the endothelium creates an environment ripe for the development of atherosclerosis and subsequently CVD and is associated with oxidative stress, an elevation in inflammatory factors and increased inflammatory gene expression (Hein et al., 2009; Kusche-Vihrog et al., 2011; Sena et al., 2013; Venugopal et al., 2002) (Bonetti et al., 2003; Sena et al., 2013). Thus, identification of those at higher risk of endothelial dysfunction may help to reduce the risk of CVD since it is the earliest aberration to be detected in most vascular diseases. There are many contributors to endothelial dysfunction including lifestyle, diet and medications but one important contributor is the aberrant metabolism of the anti-inflammatory mediator nitric oxide (Boger et al., 1997; Quyyumi et al., 1995) (Bhanoori, 2011; Brenol et al., 2010; da Costa Escobar Piccoli et al., 2012). A reduction in the synthesis and release of nitric oxide is associated with decreased inhibition of platelet aggregation, leading to inflammation, endothelial dysfunction and vascular muscle contraction (Diodati et al., 1998). Endothelial nitric oxide synthetase (eNOS) is one of the nitric oxide synthetases (NOS) responsible for the production of nitric oxide from L-arginine. Genetic variation within the endothelial nitric oxide synthetase gene (*eNOS*) may result in impaired endogenous nitric oxide formation and has been associated with cardiovascular diseases (Metzger et al., 2005; Metzger et al., 2007; Napoli and Ignarro, 2007; Sandrim et al., 2006). Two commonly studied genetic *eNOS* variants are: 1) a synonymous single nucleotide polymorphism located at the -786 position in the promoter region (T⁻⁷⁸⁶C, rs2070744) and 2) a non-synonymous single-nucleotide polymorphism in exon 7 which results in a glutamine being changed with aspartate (Glu298Asp, rs1799983). No previous studies have investigated the effect of these two *eNOS* variants on endothelial functioning in a schizophrenia sample largely exposed to atypical antipsychotics.

Taken together, since AAPs increase the risk of metabolic syndrome and CVD which, in turn, is associated with a pro-inflammatory state and a potential increase in endothelial dysfunction due to the pro-inflammatory factors interfering with nitric oxide production in this state, a pharmacogenetic investigation into genes controlling nitric oxide production in schizophrenia patients taking AAPs may prove useful in identifying patients at risk for endothelial dysfunction. Thus, the hypothesis of this candidate gene study is that the impaired nitric oxide production conferred by the *eNOS* T⁻⁷⁸⁶C and Glu298Asp variants will lead to poorer endothelial functioning in schizophrenia patients as measured by peripheral artery tonometry. Furthermore, we hypothesize that the aberrant nitric oxide metabolism caused by these polymorphisms would be influenced by the presence of the pro-inflammatory state, metabolic syndrome, due to its association with poor cardiovascular status.

Methods

Subjects

Patients were recruited from mental health clinics within the southeastern Michigan area and considered for inclusion if they had the following: 1) carried a Diagnostic and Statistical Manual of Mental Disorders-4th Edition-Text Revision (DSM-IV-TR) diagnosis of schizophrenia or schizoaffective disorder, 2) were between the ages of 18 and 90, 3) were on at least one antipsychotic and 4) had no changes to their medications in the prior 6 months. Patients were excluded if they could not give informed consent, were unwilling to participate or had an active substance abuse or dependence diagnosis (however, nicotine or caffeine users were included). This study was carried out in agreement with the declaration of Helsinki and was approved by the University of Michigan Institutional Review Board.

Clinical Assessments

A schizophrenia or schizoaffective diagnosis was confirmed by the Structured Clinical Interview for DSM Diagnoses (SCID) conducted by a trained research assistant and by secondary medical record review. An assessment of current and past medication history including over-the-counter and herbal medication usage was completed for each subject, which was also validated by medical record review. A social history including smoking history, alcohol and drug use was conducted. Study assessments were generally completed in the morning within two hours of the subject's usual waking time. Subjects were asked to fast for at least 8 hours prior to coming in and all assessments were completed at this single visit. Vital signs, body mass index and hip/waist measurements were taken for each patient. Blood samples were drawn for genetic and fasting metabolic assessments including, homocysteine, glucose, insulin, HbA1C and a complete lipid panel (Total Cholesterol (TC), triglycerides (TG), low density and high density lipoprotein (LDL, HDL)). The National Cholesterol Education Program's Adult Treatment Panel III (NCEP/ATP III-a) (Grundy et al., 2005; National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III), 2002) criteria were used to diagnose metabolic syndrome for each patient if they had three or more of the following: (i) Waistline ≥ 40 inches (men) or ≥ 35 inches (women), (ii) blood pressure ≥ 130/85 or currently being treated for hypertension, (iii) triglycerides >150mg/dL,

(iv) HDL < 50mg/dL (men) or <40mg/dL (women) or currently being treated for abnormal lipids and (v) fasting blood glucose ≥ 100mg/dL or currently being treated for elevated blood glucose. Finally, physical activity was assessed based on a previously designed questionnaire (Orrell et al., 2007) which has been validated in a population with coronary heart disease using RT3 triaxial accelerometer measurements. For this assessment participants are asked about their total amount of time (in minutes/week during the week immediately preceding the study visit where DNA was obtained) spent engaging in 'strenuous activity' (e.g., jogging, aerobics), 'moderate activity' (e.g., housework, light jogging, painting and so on), and 'mild activity' (e.g., walking). A "Total Activity Score" (in metabolic equivalent of task (MET) per min) was computed by multiplying the time for each activity (in min) by a metabolic equivalent score (that is, 3, 5 or 7 METs).

Endothelial Function Assessment

Endothelial function was assessed using the peripheral artery tonometry (RH-PAT) method via the EndoPat 2000 device (Itamar Medical Inc, Caesarea, Israel). Briefly, this assessment requires patients to sit still for a minimum of 15 minutes with a digital probe on each index finger and a blood pressure cuff on their non-dominant arm. The assessment is broken up evenly into three periods (e.g., three 5 minute periods) and for the middle period the cuff is filled with a pressure of 200mmHg or 60mmHg above the subject's blood pressure. RH-PAT values are calculated using the algorithm provided by Itamar Medical Inc EndoPat2000 software. The RH-PAT value is a calculation of the post-to-pre occlusion endopat signal in the occluded arm, compared to the same ratio in the control arm and controlled for baseline vascular tone. This clinically useful method of measuring endothelial-dependent vasodilation has been shown to be reflective of nitric oxide bioavailability. It has been validated and previously described within the several populations (Bonetti et al., 2004; Ellingrod et al., 2011; Rubinshtein et al., 2010).

Genetic Analysis

DNA was extracted from whole blood samples using the salt precipitation method (Lahiri and Nurnberger, 1991). Genotyping for both the *eNOS* T⁻⁷⁸⁶C (DBSNP rs2070744) and Glu298Asp (DBSNP rs1799983) polymorphisms was completed using pyrosequencing methods (Marsh et al., 2005). Pyrosequencing primers were designed using Pyromark Assay Design Software Version 2.0 (Qiagen Group, USA). Specific conditions for each variant's assay are available upon request.

Statistical Analysis

Subjects were considered atypical antipsychotic (AAP) users if they were currently taking olanzapine, clozapine, quetiapine, risperidone, iloperidone, paliperidone, ziprasidone or aripiprazole. Furthermore, antipsychotics were broken into groups based on their propensity to cause metabolic side effects (see Table 1). Cumulative chlorpromazine equivalents (CPZE) were standardized by converting dosage regimens to chlorpromazine equivalents based on a mg/kg basis then adding equivalents together if antipsychotic polypharmacy was present (Andreasen et al., 2010). In order to determine any baseline differences based on genotype, student t-tests were used to compare the mean values of demographic

characteristics and chi-squared analysis was used to compare dichotomous variables. To assess the association between each *eNOS* genotype and RH-PAT values, ANOVA and regression analysis were conducted on the entire schizophrenia sample. The same analyses were conducted after stratifying the sample by presence or absence of metabolic syndrome. The regression analyses used RH-PAT index as the dependent variable and antipsychotic exposure (cumulative CPZE), smoking and race as independent variables since these differed by genotype or affected RH-PAT values (detailed in results below). All analyses were conducted using the JMP 9.0 statistical program. A p-value <0.05 with a 95% confidence interval was considered significant for this study.

Results

Demographic Analysis

Our population included 203 males and females with schizophrenia (59.1%) or schizoaffective disorder (40.9%). The average age of our sample was 46.0 ± 11.5 years. Approximately 36% were female, 56.7% white and 35.5% African American which is a natural reflection of the recruitment area. The rest of the population was American Indian, Asian, Hispanic or Latino. A large majority of the sample used AAPs (85.7%) while the rest used one of the following first generation antipsychotics; haloperidol, fluphenazine, perphenazine or trifluoperazine. Our population's rate of metabolic syndrome (36.5%) is similar to that of other studies (McEvoy et al., 2005; Meyer et al., 2005) and of note, Total Activity Scores did not differ based by metabolic syndrome stratification ($p=0.2$). The *eNOS* T^{-786C} and Glu298Asp variants were determined to be in Hardy-Weinberg equilibrium (both $p>0.09$) and not in linkage disequilibrium ($D'=0.471$) using Haploview Version 4.2. We were unable to genotype 5 participants for the *eNOS* T^{-786C} variant and 6 participants for the *eNOS* Glu298Asp variant due to sample fatigue. For population demographics as well as demographics stratified by metabolic syndrome see Table 1.

Genotype Group Comparisons

Preliminary analysis to identify differences based on genotype showed one difference. Both variants had significant differences when looking at race (both $p<0.001$). The *eNOS* T^{-786C} polymorphism had no African-Americans with the CC genotype and the *eNOS* Glu298Asp had 2 African-Americans with the AspAsp genotype. Please see Table 2 for a breakdown of each variant by race.

RH-PAT and Demographic Variables

Similar to our previous work looking at variables affecting RH-PAT index; age, race, gender, cumulative CPZE, AAP use or smoking status did not significantly affect endothelial function (smoking status $p=0.07$ and all others $p>0.15$). When stratifying by presence of NCEP/ATP III-a metabolic syndrome, it was found that RH-PAT index was significantly affected by smoking status ($F(1,115)=5.12$, $p=0.02$) and cumulative CPZE ($F(1,110)=5.77$, $p=0.01$) only in those subjects *without* metabolic syndrome. Finally, RH-PAT did not differ by Total Activity Score prior to or after stratifying by metabolic syndrome ($p>0.7$ for all).

Genotype and RH-PAT

We sought to determine the overall pharmacogenetic effect of the *eNOS* variants on RH-PAT index within our collective schizophrenia cohort. Both variants showed no significant association with RH-PAT index ($T^{-786}C$: $F(2,177)=2.3$, $p=0.1$ and $Glu298Asp$: $F(2,176)=1.72$, $p=0.2$) when looking at the group as a whole. The *eNOS* $T^{-786}C$ variant showed a trend (Cohen's effect size $d=0.5$) for the CC genotype to have a higher RH-PAT $\pm$ s.e. (2.03 ± 0.13) versus the TT genotype (RH-PAT $\pm$ s.e. $=1.75 \pm 0.06$), which may be of clinical significance as a value of 1.67 has been identified as a threshold for meeting criteria for endothelial dysfunction (Bonetti et al., 2004). Next we stratified our sample by presence of NCEP/ATP III-a metabolic syndrome. We found a significant correlation of RH-PAT index with the *eNOS* $T^{-786}C$ variant in those participants without metabolic syndrome where the CC genotype had a mean $\pm$ s.e. RH-PAT of 2.17 ± 0.163 and the TT genotype had a mean $\pm$ s.e. RH-PAT of 1.71 ± 0.069 ($F(2,113)=3.41$, $p=0.03$, Cohen's effect size $d = 0.8$). In those participants with metabolic syndrome the RH-PAT and $T^{-786}C$ correlation was not present ($F(63,2)=0.057$, $p=0.9$). There were no significant associations between RH-PAT and the *Glu298Asp* polymorphism for either participants with or without metabolic syndrome ($p=0.6$ and $p=0.3$, respectively). Please see Table 3 and Figure 1 comparisons of RH-PAT index and endothelial dysfunction based on the *eNOS* $T^{-786}C$ variant and presence of metabolic syndrome.

Given the effect of antipsychotic exposure (cumulative CPZE) and smoking status on endothelial function (Alian et al., 2012; Guo et al., 2009), as well as the racial differences seen in the variants within our sample (Tanus-Santos et al., 2001), we performed a regression analysis where RH-PAT was the dependent variable and cumulative CPZE, race, smoking status and *eNOS* genotype were used as the independent variables. Our regression analysis on the collective schizophrenia cohort (regardless of metabolic syndrome diagnosis) yielded no significant association with RH-PAT index (both >0.2). However, when stratifying by presence of metabolic syndrome, our regression showed a significant association where the *eNOS* $T^{-786}C$ variant ($F=3.65$, $p=0.02$) and smoking status ($F=6.03$, $p=0.01$) for those *without metabolic syndrome* contributed to the significance of the whole model ($F(6,108)=3.39$, $p=0.004$). In contrast, the same regression model in those *with* metabolic syndrome was not significant ($F(6,62)=0.50$, $p=0.8$). When using the same model above but instead looking at the *eNOS* *Glu298Asp* variant, the model was significant ($F(6,106)=2.50$, $p=0.02$) in those patients without metabolic syndrome due to the effect of smoking ($F=4.10$, $p=0.04$) and cumulative CPZE ($F=4.81$, $p=0.03$) but not genotype ($F=0.9$, $p=0.4$). The regression analysis was non-significant when looking at the *Glu298Asp* variant in schizophrenia patients with metabolic syndrome ($F(6,63)=0.5$, $p=0.8$).

Discussion

Overall, approximately 48% of our population met the criteria for endothelial dysfunction (RH-PAT <1.67 , see Table 1) (Bonetti et al., 2003). Given the known adverse effects of metabolic syndrome on the cardiovascular system (Ingelsson et al., 2007) it is noteworthy that those meeting metabolic syndrome criteria did not have a significantly higher percentage meeting endothelial dysfunction compared to those without metabolic syndrome

(49% vs. 47%, $p=0.8$) nor was there a significant difference in RH-PAT values ($p=0.9$). This finding does not take either of the *eNOS* variants into account. However, segregating our schizophrenia subjects based on presence of metabolic syndrome did reveal variables that influence RH-PAT index including cumulative CPZE and smoking status in subjects without metabolic syndrome. Both smoking status and atypical antipsychotics (which a majority of our subjects were on) are known risk factors for cardiovascular side effects (Grassi et al., 2010; Puranik and Celermajer, 2003), but it appears that these factors do not play a significant role in endothelial function in schizophrenia patients who progress to meet metabolic syndrome criteria. Furthermore, our regression analyses found that schizophrenia participants that do not have metabolic syndrome had significantly worse RH-PAT values if they carried the TT genotype of the *eNOS* T⁻⁷⁸⁶C variant which contradicts studies in healthy subjects using brachial artery function as a predictor of endothelial function (Imamura et al., 2008). However, this study did not directly report the rate of metabolic syndrome in their population.

Taking our results together, once metabolic syndrome is present, the adverse effects on the cardiovascular system may be creating a state where endothelial dysfunction in schizophrenia is not significantly affected by smoking, CPZE status or *eNOS* genotype. A possible explanation for this occurrence could be the pro-inflammatory state created by metabolic syndrome and its associated insulin resistance which is driving the endothelial dysfunction and overriding any genetic protection with the *eNOS* variants' production of nitric oxide (Leonard et al., 2012). Previous studies have correlated elevations in inflammatory markers with metabolic syndrome in various populations (Grundy et al., 2005; Koh et al., 2005; Lee et al., 2000; Prossin et al., 2013). Indeed, there were expected differences when comparing schizophrenia participants with metabolic syndrome and without metabolic syndrome. Specifically, there were differences in either the criteria that defines metabolic syndrome (e.g., triglycerides, BMI, fasting glucose) or known factors that increase the risk of getting metabolic syndrome like age, sex and type of antipsychotic currently being taken. The culmination of these various differences within these two groups create the distinct metabolic syndrome phenotype that exposes certain variables like smoking, cumulative CPZE and genotype that influence RH-PAT index when it is not present and overrides these variables' influence on RH-PAT index when it is present. It may be possible that the *eNOS*-786CC variant of nitric oxide, a gaseous anti-inflammatory mediator of the inflammatory cascade, is only able to preserve endothelial functioning in schizophrenia patients that are not subject to the pro-inflammatory factors associated with metabolic syndrome. However, when schizophrenia patients progress to metabolic syndrome, the endothelial function is no longer responsive to genetic protection of nitric oxide variants investigated in this study but rather the inflammatory cascade that results from this syndrome. This theory could be a future line of research in endothelial dysfunction in schizophrenia.

The RH-PAT-genotype differences seen between those with and without metabolic syndrome may be a result of the adequate treatment of the individual criteria of metabolic syndrome (insulin resistance, lipid abnormalities, etc) as there is a line of research purporting the anti-inflammatory effects of hydroxymethylglutaryl CoA reductase inhibitors

(statins) (Antonopoulos et al., 2012; Balakumar et al., 2012). As would be expected, the use of statins was more prevalent in the group with metabolic syndrome than without (55% versus 12%, respectively) however RH-PAT values were not influenced by statin use ($p=0.6$). Given that many schizophrenia patients taking atypical antipsychotics progress to metabolic syndrome, the factors that are influencing endothelial dysfunction in these patients warrant further investigation. The findings in this study add to the plethora of research showing the detrimental consequences of atypical antipsychotic associated metabolic syndrome and the need to find personalized medicine or alternate interventions in order to prevent and lessen this comorbidity in the schizophrenia population.

Our study is not the first to find associations with *eNOS* variants and a cardiovascular phenotype that depended on the presence of metabolic syndrome. A study by Pons et al. looking at restenosis in patients that have undergone percutaneous coronary intervention found an opposite interaction between metabolic syndrome and *eNOS* variants (Pons et al., 2009). Their study discovered higher rates of clinical restenosis which varied depending on *eNOS* variant in patients *with* metabolic syndrome only. The variants did not have a significant effect on clinical restenosis in patients without metabolic syndrome. This study used the patient population from the Genetic Determinants of Restenosis (GENDER) study (Agema et al., 2004) which only takes cardiovascular and lipid-lowering medications into account but does not account for any other medications that may affect metabolic syndrome rates like atypical antipsychotics, making direct comparisons to our unique population difficult.

Within mental illness, there has been one other study looking at peripheral arteriole endothelialdependent vasodilation in unmedicated schizophrenia patients and controls (Israel et al., 2011). This study did not have a genetic component and used a different measure of endothelial function (laser Doppler flowmetry, LDF) therefore their brachial measurements results cannot be compared to our digital measurements since both provide distinct information (Hamburg et al., 2011). Nevertheless, this group found evidence of peripheral endothelial dysfunction in patients with acute schizophrenia not yet treated with medications compared to healthy controls. Given the link between endothelial dysfunction in both unmedicated and medicated schizophrenia patients found in studies completed to date, future prospective studies investigating the link between endothelial dysfunction and cardiovascular events are needed. An emphasis on assessing additive endothelial dysfunction risk, if any, on treating schizophrenia patients with atypical antipsychotics should also be investigated.

There are a few limitations that need to be addressed when interpreting the results of our study. This study was cross-sectional in nature with a limited sample size and therefore the results need to be repeated in separate, prospective samples to confirm the effects of the *eNOS* polymorphisms on endothelial function in schizophrenia. It would also be highly useful to correlate the results with a measure of nitric oxide and inflammatory mediators such as C-reactive protein or an interleukin however, studies have shown correlations between endothelial dysfunction and elevations in inflammatory factor levels and inflammatory gene expression (Hein et al., 2009; Kusche-Vihrog et al., 2011; Sena et al., 2013; Venugopal et al., 2002). We did not investigate another common variant of the

endothelial nitric oxide synthetase gene called intron 4a4b VNTR and so associations between this variant and endothelial functioning cannot be ruled out. Our study also had multiple comparisons which we did not control for but given the significance of our main, hypothesis-driven finding, a more stringent p-value cutoff (e.g. $p < 0.01$) could be applied. Our cohort included a wide age range for inclusion and age has been shown to effect endothelial function (Celermajer et al., 1994; Egashira et al., 1993) however, we did not control for age in our regression analysis since within our sample there was no difference in RH-PAT values based on age ($p = 0.9$) and our cohort age range was relatively narrow (46.0 ± 11.5 years). Finally, although we looked at a population mainly taking metabolically adverse atypical antipsychotics, polypharmacy was common among the entire cohort.

Conclusion

This investigation found an association between the *eNOS* T⁻⁷⁸⁶C variant and endothelial functioning in a population of schizophrenia patients taking atypical antipsychotics who did not meet criteria for metabolic syndrome. However, this association was lost once the criteria for metabolic syndrome was met. Our findings suggest that schizophrenia patients may lose the genetic protection conferred by the anti-inflammatory mediator nitric oxide on endothelial function measurements once they progress to the pro-inflammatory state of metabolic syndrome. However, our findings cannot be extended to show that the *eNOS* gene is a risk factor for metabolic syndrome itself. These findings add further evidence to the need to find personalized medicine interventions to quell the metabolic side effects of the commonly used atypical antipsychotics and preserve any genetic protection these patients may have. One possible line of research given the findings our candidate-gene study could be L-arginine supplementation based on *eNOS* genotypes. Finally, future pharmacogenetic studies may need to take metabolic syndrome into account when investigating associations between a given cardiovascular phenotype and a gene variant.

Acknowledgments

Funding Acknowledgements

The following sources were utilized for this publication: NIMH (R01 MH082784), NIH-NCCR, GCRC Program (UL1RR024986), the Chemistry Core of the Michigan Diabetes Research and Training Center (NIH5P60 DK 20572), the Washtenaw Community Health Organization (WCHO, Ann Arbor, Michigan), the Ann Arbor Veterans Administration Hospital and the Detroit Wayne, the Ann Arbor Veterans Administration Hospital and the Detroit Wayne Detroit-Wayne County Community Mental Health Agency (DWCCMHA).

References

- Agema WR, Monraats PS, Zwinderman AH, De Winter RJ, Tio RA, Doevendans PA, et al. Current PTCA practice and clinical outcomes in The Netherlands: the real world in the pre-drug-eluting stent era. *European Heart Journal*. 2004; 25(13):1163–1170. [PubMed: 15231375]
- Alian Z, Hashemipour M, Dehkordi EH, Hovsepian S, Amini M, Moadab MH, et al. The effects of folic acid on markers of endothelial function in patients with type 1 diabetes mellitus. *Medicinski Arhiv*. 2012; 66(1):12–15. [PubMed: 22482335]
- Andreasen NC, Pressler M, Nopoulos P, Miller D, Ho BC. Antipsychotic dose equivalents and dose-years: a standardized method for comparing exposure to different drugs. *Biological Psychiatry*. 2010; 67(3):255–262. [PubMed: 19897178]

- Antonopoulos AS, Margaritis M, Lee R, Channon K, Antoniades C. Statins as anti-inflammatory agents in atherogenesis: molecular mechanisms and lessons from the recent clinical trials. *Current Pharmaceutical Design*. 2012; 18(11):1519–1530. [PubMed: 22364136]
- Balakumar P, Kathuria S, Taneja G, Kalra S, Mahadevan N. Is targeting eNOS a key mechanistic insight of cardiovascular defensive potentials of statins? *Journal of Molecular and Cellular Cardiology*. 2012; 52(1):83–92. [PubMed: 21968328]
- Bhanoori M. Endothelial nitric oxide synthase (eNOS) variants in cardiovascular disease: pharmacogenomic implications. *The Indian Journal of Medical Research*. 2011; 133:464–466. [PubMed: 21623028]
- Boger RH, Bode-Boger SM, Thiele W, Junker W, Alexander K, Frolich JC. Biochemical evidence for impaired nitric oxide synthesis in patients with peripheral arterial occlusive disease. *Circulation*. 1997; 95(8):2068–2074. [PubMed: 9133517]
- Bonetti PO, Lerman LO, Lerman A. Endothelial dysfunction: a marker of atherosclerotic risk. *Arteriosclerosis, Thrombosis, and Vascular Biology*. 2003; 23(2):168–175.
- Bonetti PO, Pumper GM, Higano ST, Holmes DR Jr, Kuvin JT, Lerman A. Noninvasive identification of patients with early coronary atherosclerosis by assessment of digital reactive hyperemia. *Journal of the American College of Cardiology*. 2004; 44(11):2137–2141. [PubMed: 15582310]
- Brenol CV, Chies JA, Brenol JC, Xavier RM. Role of endothelial nitric oxide synthase (eNOS) polymorphisms in cardiovascular disease and rheumatoid arthritis. *Clinical and Experimental Rheumatology*. 2010; 28(4):584. author reply 584-5. [PubMed: 20810039]
- Celermajer DS, Sorensen KE, Spiegelhalter DJ, Georgakopoulos D, Robinson J, Deanfield JE. Aging is associated with endothelial dysfunction in healthy men years before the age-related decline in women. *Journal of the American College of Cardiology*. 1994; 24(2):471–476. [PubMed: 8034885]
- da Costa Escobar Piccoli J, Manfredini V, Hamester FI, Bandinelli JB, Turkienicz IM, Chies JA, et al. Interaction Between Endothelial Nitric Oxide Synthase Gene Polymorphisms (–786T>C, 894G>T and Intron 4 a/b) and Cardiovascular Risk Factors in Acute Coronary Syndromes. *Archives of Medical Research*. 2012; 43(3):205–211. [PubMed: 22475779]
- Diodati JG, Dakak N, Gilligan DM, Quyyumi AA. Effect of atherosclerosis on endothelium-dependent inhibition of platelet activation in humans. *Circulation*. 1998; 98(1):17–24. [PubMed: 9665055]
- Egashira K, Inou T, Hirooka Y, Kai H, Sugimachi M, Suzuki S, et al. Effects of age on endothelium-dependent vasodilation of resistance coronary artery by acetylcholine in humans. *Circulation*. 1993; 88(1):77–81. [PubMed: 8319359]
- Ellingrod VL, Taylor SF, Brook RD, Evans SJ, Zollner SK, Grove TB, et al. Dietary, lifestyle and pharmacogenetic factors associated with arteriole endothelial-dependent vasodilatation in schizophrenia patients treated with atypical antipsychotics (AAPs). *Schizophrenia Research*. 2011; 130(1–3):20–26. [PubMed: 21504842]
- Ford ES. Prevalence of the metabolic syndrome defined by the International Diabetes Federation among adults in the U.S. *Diabetes Care*. 2005; 28(11):2745–2749. [PubMed: 16249550]
- Galassi A, Reynolds K, He J. Metabolic syndrome and risk of cardiovascular disease: a meta-analysis. *The American Journal of Medicine*. 2006; 119(10):812–819. [PubMed: 17000207]
- Gami AS, Witt BJ, Howard DE, Erwin PJ, Gami LA, Somers VK, et al. Metabolic syndrome and risk of incident cardiovascular events and death: a systematic review and metaanalysis of longitudinal studies. *Journal of the American College of Cardiology*. 2007; 49(4):403–414. [PubMed: 17258085]
- Grassi D, Desideri G, Ferri L, Aggio A, Tiberti S, Ferri C. Oxidative stress and endothelial dysfunction: say NO to cigarette smoking! *Current Pharmaceutical Design*. 2010; 16(23):2539–2550. [PubMed: 20550504]
- Grundey SM, Cleeman JI, Daniels SR, Donato KA, Eckel RH, Franklin BA, et al. Diagnosis and management of the metabolic syndrome: an American Heart Association/National Heart, Lung, and Blood Institute Scientific Statement. *Circulation*. 2005; 112(17):2735–2752. [PubMed: 16157765]

- Guo H, Chi J, Xing Y, Wang P. Influence of folic acid on plasma homocysteine levels & arterial endothelial function in patients with unstable angina. *The Indian Journal of Medical Research*. 2009; 129(3):279–284. [PubMed: 19491420]
- Hamburg NM, Palmisano J, Larson MG, Sullivan LM, Lehman BT, Vasan RS, et al. Relation of brachial and digital measures of vascular function in the community: the Framingham heart study. *Hypertension*. 2011; 57(3):390–396. [PubMed: 21263120]
- Hein TW, Singh U, Vasquez-Vivar J, Devaraj S, Kuo L, Jialal I. Human C-reactive protein induces endothelial dysfunction and uncoupling of eNOS in vivo. *Atherosclerosis*. 2009; 206(1):61–68. [PubMed: 19268941]
- Hennekens CH, Hennekens AR, Hollar D, Casey DE. Schizophrenia and increased risks of cardiovascular disease. *American Heart Journal*. 2005; 150(6):1115–1121. [PubMed: 16338246]
- Imamura A, Takahashi R, Murakami R, Kataoka H, Cheng XW, Numaguchi Y, et al. The effects of endothelial nitric oxide synthase gene polymorphisms on endothelial function and metabolic risk factors in healthy subjects: the significance of plasma adiponectin levels. *European Journal of Endocrinology / European Federation of Endocrine Societies*. 2008; 158(2):189–195. [PubMed: 18230825]
- Ingelsson E, Sullivan LM, Murabito JM, Fox CS, Benjamin EJ, Polak JF, et al. Prevalence and prognostic impact of subclinical cardiovascular disease in individuals with the metabolic syndrome and diabetes. *Diabetes*. 2007; 56(6):1718–1726. [PubMed: 17369522]
- Israel AK, Seeck A, Boettger MK, Rachow T, Berger S, Voss A, et al. Peripheral endothelial dysfunction in patients suffering from acute schizophrenia: a potential marker for cardiovascular morbidity? *Schizophrenia Research*. 2011; 128(1–3):44–50. [PubMed: 21371864]
- Koh KK, Han SH, Quon MJ. Inflammatory markers and the metabolic syndrome: insights from therapeutic interventions. *Journal of the American College of Cardiology*. 2005; 46(11):1978–1985. [PubMed: 16325028]
- Kusche-Vihrog K, Urbanova K, Blanque A, Wilhelmi M, Schillers H, Kliche K, et al. C-reactive protein makes human endothelium stiff and tight. *Hypertension*. 2011; 57(2):231–237. [PubMed: 21149827]
- Lahiri DK, Nurnberger JI Jr. A rapid non-enzymatic method for the preparation of HMW DNA from blood for RFLP studies. *Nucleic Acids Research*. 1991; 19(19):5444. [PubMed: 1681511]
- Laursen TM, Munk-Olsen T, Vestergaard M. Life expectancy and cardiovascular mortality in persons with schizophrenia. *Current Opinion in Psychiatry*. 2012; 25(2):83–88. [PubMed: 22249081]
- Lee SC, Pu YB, Thomas GN, Lee ZS, Tomlinson B, Cockram CS, et al. Tumor necrosis factor alpha gene G-308A polymorphism in the metabolic syndrome. *Metabolism: Clinical and Experimental*. 2000; 49(8):1021–1024. [PubMed: 10954020]
- Leonard BE, Schwarz M, Myint AM. The metabolic syndrome in schizophrenia: is inflammation a contributing cause? *Journal of Psychopharmacology (Oxford, England)*. 2012; 26(5 Suppl):33–41.
- Marsh S, King CR, Garsa AA, McLeod HL. Pyrosequencing of clinically relevant polymorphisms. *Methods in Molecular Biology (Clifton, N.J.)*. 2005; 311:97–114.
- McEvoy JP, Meyer JM, Goff DC, Nasrallah HA, Davis SM, Sullivan L, et al. Prevalence of the metabolic syndrome in patients with schizophrenia: baseline results from the Clinical Antipsychotic Trials of Intervention Effectiveness (CATIE) schizophrenia trial and comparison with national estimates from NHANES III. *Schizophrenia Research*. 2005; 80(1):19–32. [PubMed: 16137860]
- Metzger IF, Sertorio JT, Tanus-Santos JE. Modulation of nitric oxide formation by endothelial nitric oxide synthase gene haplotypes. *Free Radical Biology & Medicine*. 2007; 43(6):987–992. [PubMed: 17697943]
- Metzger IF, Souza-Costa DC, Marroni AS, Nagassaki S, Desta Z, Flockhart DA, et al. Endothelial nitric oxide synthase gene haplotypes associated with circulating concentrations of nitric oxide products in healthy men. *Pharmacogenetics and Genomics*. 2005; 15(8):565–570. [PubMed: 16007000]
- Meyer JM, Nasrallah HA, McEvoy JP, Goff DC, Davis SM, Chakos M, et al. The Clinical Antipsychotic Trials Of Intervention Effectiveness (CATIE) Schizophrenia Trial: clinical

- comparison of subgroups with and without the metabolic syndrome. *Schizophrenia Research*. 2005; 80(1):9–18. [PubMed: 16125372]
- Napoli C, Ignarro LJ. Polymorphisms in endothelial nitric oxide synthase and carotid artery atherosclerosis. *Journal of Clinical Pathology*. 2007; 60(4):341–344. [PubMed: 16837626]
- National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III). Third Report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III) final report. *Circulation*. 2002; 106(25):3143–3421. [PubMed: 12485966]
- Orrell A, Doherty P, Miles J, Lewin R. Development and validation of a very brief questionnaire measure of physical activity in adults with coronary heart disease. *European Journal of Cardiovascular Prevention and Rehabilitation : Official Journal of the European Society of Cardiology, Working Groups on Epidemiology & Prevention and Cardiac Rehabilitation and Exercise Physiology*. 2007; 14(5):615–623.
- Pons D, Monraats PS, Zwinderman AH, de Maat MP, Doevendans PA, de Winter RJ, et al. Metabolic background determines the importance of NOS3 polymorphisms in restenosis after percutaneous coronary intervention: A study in patients with and without the metabolic syndrome. *Disease Markers*. 2009; 26(2):75–83. [PubMed: 19407362]
- Prossin AR, Zalcman SS, Evans SJ, McInnis MG, Ellingrod VL. A pilot study investigating tumor necrosis factor-alpha as a potential intervening variable of atypical antipsychotic-associated metabolic syndrome in bipolar disorder. *Therapeutic Drug Monitoring*. 2013; 35(2):194–202. [PubMed: 23503445]
- Puranik R, Celermajer DS. Smoking and endothelial function. *Progress in Cardiovascular Diseases*. 2003; 45(6):443–458. [PubMed: 12800127]
- Quyyumi AA, Dakak N, Andrews NP, Husain S, Arora S, Gilligan DM, et al. Nitric oxide activity in the human coronary circulation. Impact of risk factors for coronary atherosclerosis. *The Journal of Clinical Investigation*. 1995; 95(4):1747–1755. [PubMed: 7706483]
- Rubinshtein R, Kuvin JT, Soffler M, Lennon RJ, Lavi S, Nelson RE, et al. Assessment of endothelial function by non-invasive peripheral arterial tonometry predicts late cardiovascular adverse events. *European Heart Journal*. 2010; 31(9):1142–1148. [PubMed: 20181680]
- Sandrim VC, de Syllos RW, Lisboa HR, Tres GS, Tanus-Santos JE. Endothelial nitric oxide synthase haplotypes affect the susceptibility to hypertension in patients with type 2 diabetes mellitus. *Atherosclerosis*. 2006; 189(1):241–246. [PubMed: 16427644]
- Sena CM, Pereira AM, Seica R. Endothelial dysfunction - a major mediator of diabetic vascular disease. *Biochimica Et Biophysica Acta*. 2013
- Tanus-Santos JE, Desai M, Flockhart DA. Effects of ethnicity on the distribution of clinically relevant endothelial nitric oxide variants. *Pharmacogenetics*. 2001; 11(8):719–725. [PubMed: 11692081]
- Venugopal SK, Devaraj S, Yuhanna I, Shaul P, Jialal I. Demonstration that C-reactive protein decreases eNOS expression and bioactivity in human aortic endothelial cells. *Circulation*. 2002; 106(12):1439–1441. [PubMed: 12234944]

Significant Outcomes

- Neither the *eNOS* Glu298Asp or the T⁻⁷⁸⁶C variant significantly affected endothelial function of schizophrenia patients when not accounting for metabolic syndrome diagnosis.
- Patients diagnosed with schizophrenia and not meeting metabolic syndrome criteria had significantly better endothelial functioning if they carried the CC genotype of the *eNOS* T⁻⁷⁸⁶C variant.

Limitations

- This study was relatively small and cross-sectional in nature and therefore the results need to be repeated in separate, prospective samples to confirm the effects of the *eNOS* polymorphisms on endothelial function in schizophrenia.
- Examination of endothelial dysfunction and the *eNOS* polymorphisms requires correlation to inflammatory markers and inflammatory gene expression in order to confirm the findings in this study
- This study did not examine another common variant of the endothelial nitric oxide synthetase gene called intron 4a4b VNTR and so associations between this variant and endothelial functioning cannot be ruled out.

Figure 1. *eNOS* $T^{-786}C$ and Endothelial Function Based on Presence of Metabolic Syndrome
 Depicts the *eNOS* $T^{-786}C$ variant and its association with endothelial function as measured by RH-PAT index. For schizophrenia subjects without metabolic syndrome, the CC genotype demonstrates superior endothelial functioning measurements as compared to the aberrant metabolism induced by the TT genotype. However, when looking at the schizophrenia individuals who have metabolic syndrome, the endothelial function is overall poorer and does not differ based on *eNOS* $T^{-786}C$ variant thus suggesting the genetic protection of the CC genotype has been lost.

Table 1

Population demographics

	Schizophrenia Spectrum Disorders (n=203)	Meeting NCEP/ATP III Metabolic Syndrome (n=74)	Does not meet NCEP/ATP III Metabolic Syndrome (n=129)
Age ± s.d. (years)	46.0±11.5	49.6 ± 9.24	43.9 ± 12.1
% Female	36.5	43.2	32.6
% Caucasian/African-American	56.7/35.5	55.4/37.8	57.4/34.1
% NCEP/ATP III Metabolic Syndrome	36.5	-----	-----
% Current Smoker	52.2	52.7	51.9
% AAP use	85.7	90.5	83.0
% using olanzapine or clozapine	27.6	37.8	21.7
% using risperidone, paliperidone, quetiapine, iloperidone	37.4	32.4	40.3
% using ziprasidone, aripiprazole or typical antipsychotic	35.0	29.7	38.0
Antipsychotic Chlorpromazine Equivalents (mg)	645 ± 584	693 ± 613	616 ± 567
hip/waist ratio	1.05 ± 0.09	1.01 ± 0.08	1.07 ± 0.09
BMI ± s.d. (kg/m ²)	32.4 ± 7.76	36.6 ± 7.74	30.0 ± 6.72
Systolic Blood Pressure ± s.d. (mmHg)	123 ± 17.3	127 ± 16.0	121 ± 17.6
Diastolic Blood Pressure ± s.d. (mmHg)	74.8 ± 12.4	76.0 ± 13.4	74.1 ± 11.7
RH-PAT ± s.d.	1.82 ± 0.5	1.81 ± 0.5	1.83 ± 0.5
% RH-PAT < 1.67	48.1	49.2	47.4
Homocysteine ± s.d. (umol/L)	11.1 ± 4.5	11.1 ± 3.7	11.1 ± 4.9
Glucose ± s.d. (mg/dL)	105 ± 47.2	116 ± 65.0	98.6 ± 30.5
HbA1C ± s.d	6.04 ± 1.29	6.55 ± 1.70	5.74 ± 0.85
Total Cholesterol ± s.d. (mg/dL)	170 ± 41.4	171 ± 45.4	170 ± 38.9
Triglycerides ± s.d. (mg/dL)	134 ± 96.8	181 ± 121	105 ± 65.0
High Density Lipoprotein ± s.d. (mg/dL)	51.7 ± 16.0	46.4 ± 13.0	54.9 ± 16.8
Low Density Lipoprotein ± s.d. (mg/dL)	103 ± 32.3	101 ± 34.7	104 ± 31.0
Total Activity Score (MET/min)	2578 ± 2552	2284 ± 1848	2745 ± 2870
% ENOS -786 TT/TC/CC	45.0/45.0/10.0	37.0/50.7/12.3	49.6/41.6/8.8
% ENOS 298 GluGlu/GluAsp/AspAsp	57.9/33.0/9.14	54.1/37.8/8.1	60.2/30.1/9.7

Table 2

eNOS Variant by Race and Folate Status

<i>eNOS</i> Variant	Caucasian % (n)	African-American % (n)	Other* % (n)	P-value For Racial Differences
<i>T-786C</i>	TT	65(45)	63(10)	<0.001
	TC	54(61)	25(4)	
	CC	16(18)	12(2)	
<i>Glu298Asp</i>	GluGlu	40(45)	81(13)	<0.001
	GluAsp	46(51)	19(3)	
	AspAsp	14(16)	0	

* Other includes American Indian, Asian, Hispanic or Latino

Table 3

RH-PAT analyses for T-786C genotype

<i>eNOS</i> Variant	RH-PAT $\pm$ s.e.	F statistic and P-value	% with endothelial dysfunction (RH-PAT < 1.67)	Chi-square p-value
<i>T-786C</i> without Metabolic Syndrome	TT	F(2, 113) = 3.41, p = 0.03	55.6	$\chi^2 = 2.7$, p = 0.2
	TC		38.9	
	CC		5.5	
<i>T-786C</i> AND Metabolic Syndrome	TT	F(2, 63) = 0.05, p = 0.9	32.3	$\chi^2 = 1.6$, p = 0.4
	TC		58.0	
	CC		9.7	