THE PENNSYLVANIA STATE UNIVERSITY
SCHREYER HONORS COLLEGE

DEPARTMENT OF VETERINARY AND BIOMEDICAL SCIENCES

# SUPPRESSION OF NPC1L1 AND INFLAMMATORY CYTOKINES BY FLAVONOID COMPOUNDS

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# ABSTRACT

Aryl hydrocarbon receptor (AHR) is a ligand-activated transcription factor that is known for its ability to mediate responses to a wide variety of ligands, both exogenous and endogenous. Of particular interest is the ability of flavonoids, a naturally occurring compound ubiquitous in plants, to bind to this receptor. Due to their physiologically relevant concentrations in our diet, it is particularly important that we have a solid understanding of the short and long-term effects of its consumption.

Flavonoids have long been associated with the modulation of cholesterol levels and the inflammatory response. However, the mechanism of action for these flavonoid-mediated effects is still not clear. Since certain abundant flavonoids are ligands of AHR, we hypothesized that cholesterol homeostasis and the attenuation of the inflammatory response is at least partially regulated by AHR. Furthermore, we hypothesized that the mechanism behind cholesterol homeostasis involves repression of Niemann-pick C1-like 1 (NPC1L1), an intestinal/hepatic cholesterol transport protein that is regulated by AHR.

While we confirmed that flavonoids inhibited the expression of NPC1L1, this did not translate into any significant decreases in cholesterol absorption through NPC1L1. Furthermore, although the data suggests that flavonoids inhibit expression of inflammatory cytokines, AHR appears to have little to no role in this response.

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# Introduction

## Aryl Hydrocarbon Receptor (AHR)

The aryl hydrocarbon receptor (AHR) is a ligand-activated transcription factor that mediates the expression of a vast array of genes. Studies have identified a wide variety of synthetic or naturally occurring, exogenous substances that bind to this receptor, which include chemicals found in pesticides, vegetables and industrial products.[1,2] Many of these ligands are halogenated aromatic hydrocarbons (HAH) and polycyclic aromatic hydrocarbons (PAH), although they can diverge from this trend.[1,2] One of the more renowned ligands, due to its high toxicity, long half-life, and high affinity for the receptor, is 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD or dioxin).[1] It was present as a contaminant in herbicide sprayed during the Vietnam War to defoliate forest; employees involved in herbicide production have developed chloroacne.[1] Studies in mice have shown that TCDD, through the AHR, can damage immune function, reproduction, organ repair and lipid/glucose metabolism, along with inducing tumor formation.[3]

The broad physiological damage caused by AHR is due to its huge battery of target genes. AHR is known to induce phase I and phase II drug-metabolizing enzymes like, CYP1A1.[4] These enzymes are important in converting substrates to a more easily excretable state, they can also metabolize substrates to a more toxic state. Several studies have also shown that benzo[a]pyrene, another AHR ligand, cause the activation of carcinogenic metabolites.[4] Among many others, AHR also triggers a number of signaling cascades that up regulate expression of genes needed for progression through the cell cycle.[5] These include genes from the Fos and Jun families, MYC and MYB.[5]

At the same time, despite the known damage caused by AHR-mediated gene expression, studies using *Ahr*-knockout mice have shown that AHR is important in the development of organ systems, like the liver and the immune system.[1] It has been noted that low levels of exposure to AHR agonists can

downregulate the inflammatory response of stromal cells toward bacterial lipopolysaccharide by repressing NFκB-mediated transcription of IL-6.[7] AHR has also been observed to repress AP-1 DNA-binding activity.[8] Furthermore, an absence of AHR leads to increased expression of TNF-α and IL-6 in the lungs in response to cigarette smoke.[9] All this evidence suggests that AHR has beneficial physiological purposes. Due to the AHR's ability to bind to a variety of ligands and target a wide array of genes for beneficial and detrimental functions depending on the context of exposure, it is important that the activation of the AHR is better understood.

**AHR Structure**

The AHR is basic helix-loop-helx/Per-Arnt-Sim (bHLH/PAS) transcription factor.[10] It is composed of three regions. It has a bHLH motif at the N-terminal, which allows for binding to DNA and the heterodimerization of AHR and ARNT.[11] It also has a PAS domain and a highly variable glutamine-rich region near the C-terminal.[11] The PAS domain, which is a necessity for protein-protein interaction and ligand binding, consists of two degenerate direct repeats, PAS A and PAS B.[12] The glutamine-rich region is required for transcriptional activation purposes.[11] The functional domains of AHR are shown in Figure 1.

It is also interesting to note that there are differences in AHR amino acid sequences between different species. This translates into distinct differences in sensitivity and endpoints that are not consistent between species.[10] For example, a lethal dose of TCDD for guinea pigs ($LD_{50}$ ~ 1μg/kg) is several orders of magnitude lower than the golden Syrian hamster ($LD_{50}$ ~ 5000μg/kg).[10]

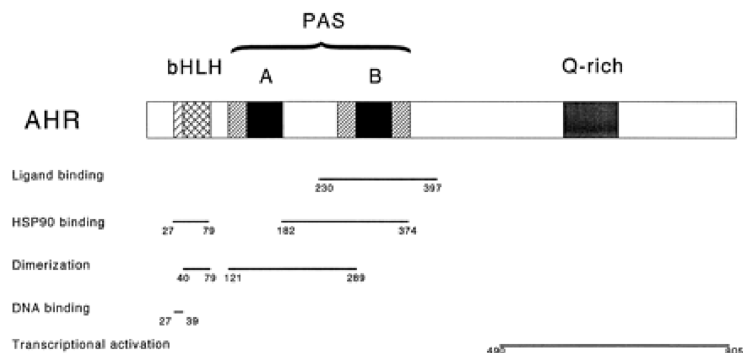


**Figure 1: Functional Domains of AHR[13]**

## Classical Mechanism of AHR

In its inactivated state, AHR is found in a heterotetrameric cytosolic complex with HSP90, XAP2 and p23.[14] Ligand binding causes a conformational change that exposes a nuclear localization signal, which results in the translocation of AHR into the nucleus. This is followed by AHR dissociation from the complex and subsequent heterodimerization with the AHR nuclear translocator protein (ARNT), another bHLH-PAS protein.[14] The AHR/ARNT heterodimer binds to dioxin responsive element (DRE) sequences, activating downstream genes.[14] One of the targeted genes expresses the AHR repressor (AHRR), which inhibits AHR function by competing with AHR for ARNT.[15] A visual representation of this mechanism is shown in Figure 2 below.

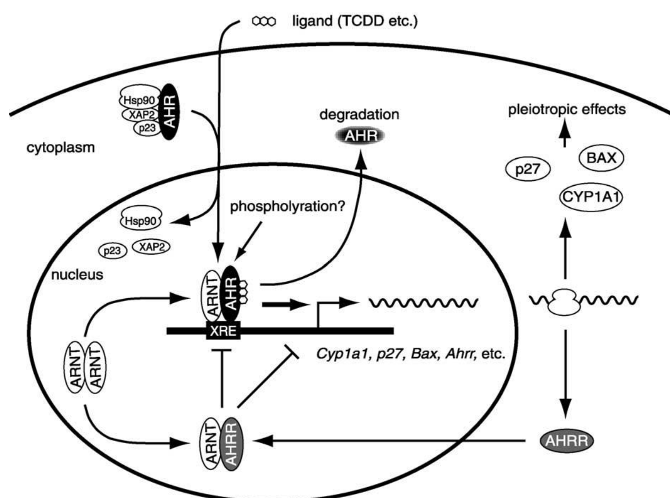


**Figure 2: Classical Mechanism of AHR[6]**

**AHR Ligands**

AHR ligands of the highest affinity tend to be hydrophobic, planar and of a certain size.[2] The vast majority of these high affinity ligands are HAHs, PAHs and other similar compounds.[2] However, due to the promiscuity of its binding pocket, AHR ligands tend to be very diverse in structure at the expense of a lower affinity for the receptor.[2] While there are no known high affinity endogenous ligands, studies have suggested that there are endogenous ligands for AHR.[16] This was initially indirectly supported by cell culture and animal model observations demonstrating AHR activation and AHR-dependent responses without the presence of exogenous ligands.[17,18] Later studies have identified indoles, tetrapyroles and arachidonic acid metabolites as possible endogenous ligands although they are weak ligands in comparison to TCDD.[16]

One of the biggest source of exposure to AHR ligands comes from our diets. Indoles, flavonoids, and carotinoids, which are compounds found in vegetables, are a few examples of such ligands.[19] These compounds are in our diets in such quantities that some are detectable in our blood as glycosides in the low μM range.[20] Furthermore, even though many of them are poor AHR ligands, some of these have the potential to become even more potent ligands when digested and undergo condensation reactions in the gut.[21] Indole-3-carbinol (I3C) upon exposure to stomach acid is converted into indolo[3,2-b]carbazole (ICZ). ICZ has a Kd of 190 pM compared to I3C which has a Kd of 22 nM-90 nM.[21]

**AHR and Cholesterol Homeostasis**

Cholesterol has many important biological functions in any vertebrate. At the same time, hypercholesterolemia carries its own set of risks, which includes promotion of atherosclerosis. Due to the dangers of having insufficient or excessive quantities of cholesterol, it is clear that cholesterol homeostasis must be maintained. There are two major sites of control for cholesterol homeostasis: the liver and the intestine.[22] The liver synthesizes cholesterol, manages the balance between low density

lipoproteins and high density lipoproteins and secretes cholesterol into bile.[22] Meanwhile, the intestine controls cholesterol absorption and secretion.[23]

There have been multiple studies that looked into the relationship between AHR and cholesterol homeostasis. A microarray study involving mice suggested that low levels of TCDD modulated the expression of genes involved in circadian rhythm, cholesterol biosynthesis, fatty acid synthesis and glucose metabolism in the liver.[24] Exposure to higher levels of TCDD led an alteration of genes involved in cholesterol metabolism and bile synthesis.[25] The mouse data was consistent with the trends seen in humans. Humans exposed to TCDD also have damaged lipid metabolism and high cholesterol/triglyceride levels.[25] While much of the mechanism still has to be elucidated, it is clear that AHR plays a significant role in cholesterol homeostasis.

**AHR and the Inflammatory Response**

The inflammatory response is a systemic reaction by the organism to a local homeostatic imbalance caused by infection, tissue injury  or immunological disorders.[26] It involves a release of pro-inflammatory cytokines at the specific site, which results in the production of more cytokine production and release into the area and its surroundings.[26] The three major cytokines released are tumor necrosis factor alpha (TNF-α), interleukin-1 (IL-1β) and interleukin-6 (IL-6).[26] While acute  inflammation has beneficial purposes, chronic inflammation has been linked to a number of health problems, including cancer.[27]

Ligand-activated AHR has been implicated as a major role player in the enhancement of inflammation..[28] Studies have shown that AHR can mediate cytokine production through the binding of the AHR/ARNT complex to. Antagonizing AHR has been shown to inhibit inflammatory cytokine production, specifically IL-6.[29]

**Overview of Research**

Flavonoids are compounds that are ubiquitous in plants and are present in physiologically relevant concentrations in our diet.[30] Thus, it is important that we have a solid understanding of the benefits and consequences of its consumption. Dietary intake of flavonoid compounds as a whole has been associated with decreased risk of cardiovascular disease and hypercholesterolemia.[31,32]

Some research papers have already suggested that flavonoid compounds, like quercetin and isorhamnetin, play a beneficial role in lipid metabolism, by decreasing the total serum concentration of cholesterol in mouse models.[31] Likewise, studies have shown that flavonoids are associated with the modulation of pro-inflammatory gene expression like, cyclooxygenase-2.[32] However, the mechanisms behind this have not been clearly elucidated. Flavonoids are known AHR ligands while AHR has an endogenous role in regulating cholesterol biosynthesis and inflammatory signalling. Thus, we hypothesize that flavonoids partially regulate cholesterol homeostasis and the inflammatory response through the AHR. Furthermore, we hypothesize that the mechanism behind cholesterol homeostasis involves repression of Niemann-pick C1-like 1 (NPC1L1), an intestinal/hepatic cholesterol transport protein that absorbs cholesterol into the system.[2, 33]

## Materials and Methods

**Cell Culture**

MCF-7 and Caco-2 lines were cultured at 37°C and 5% $CO_2$ in α-modified essential media (Sigma, St. Louis, MO) that was supplemented by 10% fetal bovine serum (Hyclone Laboratories, Logan, UT), 1000 units/mL penicillin (Sigma) and 0.1 mg/mL streptomycin (Sigma).

**RNA Extraction and Quantification**

RNA was isolated from cell culture by using TRIzol (Invitrogen, Carlsbad, CA). cDNA was created from the isolated RNA by using high-capacity cDNA archive kit (Applied Biosystems, Foster City, CA). The cDNA was quantified via a MyiQ real-time PCR system (Bio-Rad Laboratories, Hercules, CA), utilizing SYBR Green reagent (Quanta Biosciences, Gaithersburg, MD). Quantified mRNA expression was normalized to m*L13A* or *β-actin* levels.

**Cholesterol Uptake Quantification**

Cell-based cholesterol uptake was observed by utilizing a Caymen cholesterol uptake kit (Cayman, Ann Arbor, MI), which utilizes fluorescently tagged cholesterol. Cholesterol fluorescence was quantified by using Promega Glowmax multidetection system (Promega, Madison, WI.)

**Data Analysis**

All experiments were done in triplicate and analyzed by using Tukey's multiple comparison test in Prism 5 (GraphPad Software Inc., San Diego, CA). Statistical significance between different groups is indicated by asterisks (*, $P < 0.05$, **, $P < 0.01$, and ***, $P < 0.001$.)

# Results

## Quercetin and isorhamnetin are antagonists of AHR

CYP1A1 expression is primarily regulated by AHR. As such, CYP1A1 activity was used as a measure of AHR activity. To confirm that flavonoids, quercetin and isorhamnetin, are antagonists of agonist-mediated AHR transcriptional activity, MCF-7 cell line was pre-treated with kynurenic acid, an AHR agonist, and then exposed to the flavonoids and GNF351, a pure AHR antagonist.[34] CYP1A1 mRNA was quantified and analyzed. The results suggest that quercetin and isorhamnetin are AHR antagonists. Furthermore, quercetin is a weaker competitor for the AHR in comparison to isorhamnetin.

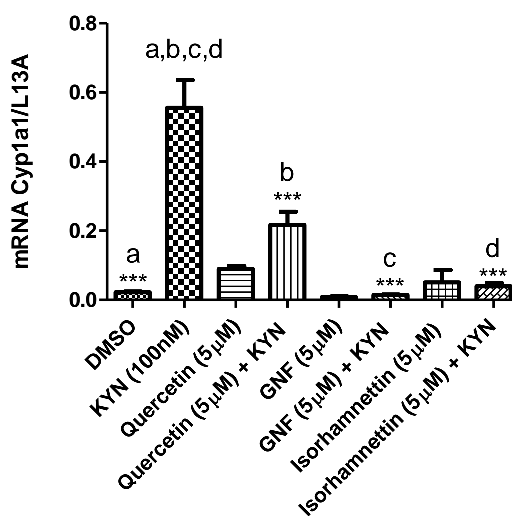


**Figure 3: Quercetin and isorhamnetin are antagonists of AHR**

MCF-7 cell line was exposed to flavonoid compounds and GNF after 1-h pretreatment with kynurenic acid (KYN), an agonist of AHR.. Cells were incubated for 4 h and CYP1A1 mRNA expression was analyzed via real-time PCR. Statistical significance is indicated by an asterisk (*, $P < 0.05$, **, $P < 0.01$, and ***, $P < 0.001$).

**Expression of NPC1L1 and Cholesterol Absorption**

The capacity of the flavonoids to repress NPC1L1 to physiologically significant levels was determined by using real-time PCR and a cholesterol uptake assay, utilizing fluorescently tagged cholesterol. Lovastatin, a drug used to regulate high cholesterol, was utilized in the cholesterol uptake assay in order to increase expression of NPC1L1. While isorhamnetin inhibited NPC1L1 mRNA expression, this did not translate into any physiological significance in the fluorescently-tagged cholesterol assay. There was no difference in raw fluorescence between the control and the other compounds. This either suggests that the level of mRNA inhibition is not physiologically significant enough or that the cell line does not produce enough of the NPC1L1 protein to note small differences in inhibition. Considering that there was no significant difference in fluorescence between the cells with lovastatin and those without, it is more likely that it is the latter.

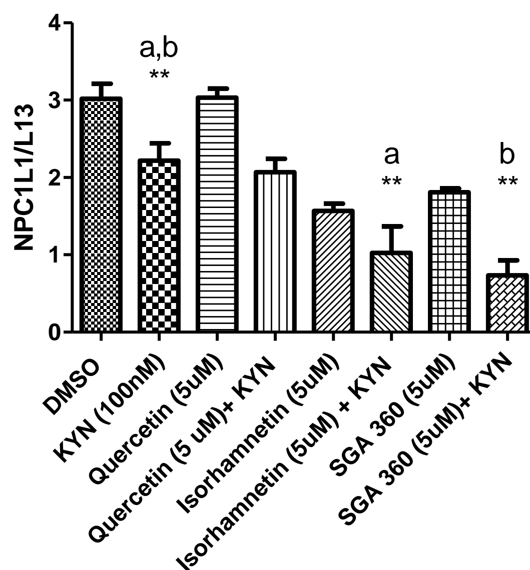


**Figure 4: Isorhamnetin decreases expression of NPC1L1**

Caco-2 cell line was exposed to flavonoid compounds after 1-h pretreatment with KYN. Cells were incubated for 4 h and NPC1L1 mRNA expression was analyzed via real-time PCR. Statistical significance is indicated by an asterisk (*, P < 0.05, **, P < 0.01, and ***, P< 0.001).

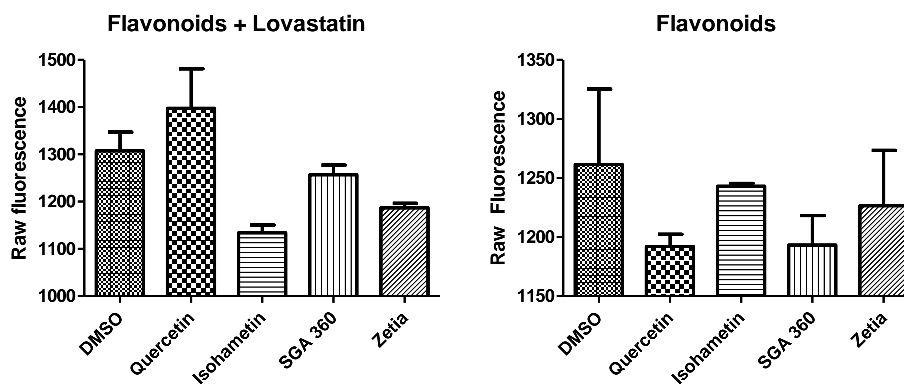


**Figure 5: Flavonoids do not inhibit cholesterol absorption via NPC1L1**

Caco-2 cell line was exposed to flavonoids or flavonoids and lovastatin for 48h. Fluorescent-tagged cholesterol was added at 24h. Cells were incubated for 48 h and raw fluorescence was quantified via a luminometer. Statistical significance is indicated by an asterisk (*, P < 0.05, **, P < 0.01, and ***, P< 0.001).

**Expression of Inflammatory Cytokines**

The capability of the flavonoids to inhibit two key inflammmatory cytokines through modulation of AHR activity was gauged using real-time PCR analysis of mRNA levels from primary macrophages isolated from wild-type AHR, $AHR^d$ and AHR-null mice. The flavonoids inhibited IL-1β and IL-6 expression to a greater extent than SGA360, a compound that exhibits no agonist activity yet exhibits strong anti-inflammatory properties in vivo.[35] However, there was no decrease in inhibition of IL-1β when the flavonoids were used on macrophages that express a low affinity AHR or a "knock-out" AHR. This suggests that the flavonoids' anti-inflammatory properties are not mediated by AHR.

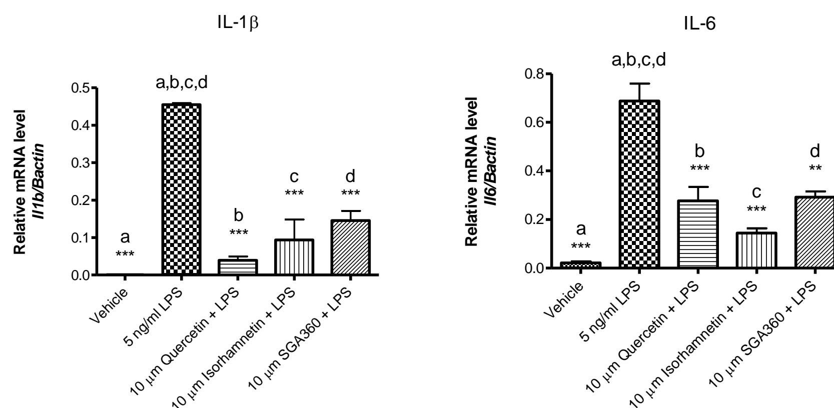


**Figure 6: Flavonoids reduce expression of inflammatory cytokines**

Primary macrophages were isolated from wild-type AHR mice. After 1-h pretreatment with LPS, the macrophages were exposed to flavonoid compounds. Cells were incubated for 4h and mRNA expression was analyzed via real-time PCR. Statistical significance is indicated by an asterisk (*, $P < 0.05$, **, $P < 0.01$, and ***, $P< 0.001$.)

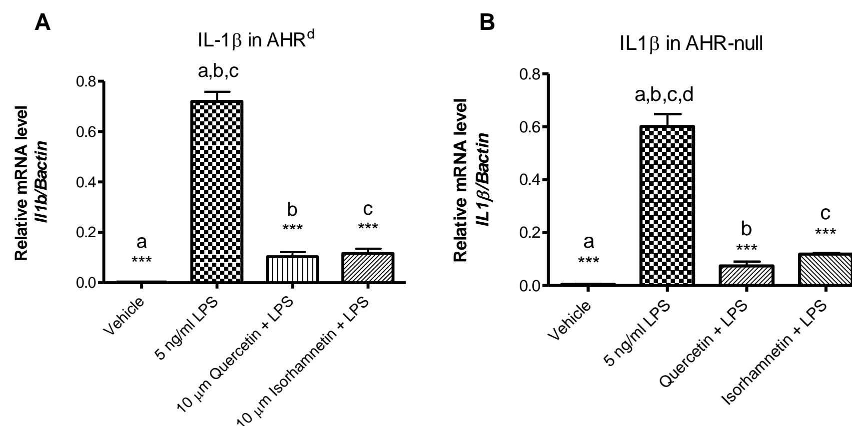


**Figure 7: Flavonoid repression of inflammatory cytokines is not mediated by AHR**

Primary macrophages were isolated from $AHR^d$ (A) and AHR-null mice (B). After 1-h pretreatment with LPS, the macrophages were exposed to flavonoid compounds. Cells were incubated for 4h and mRNA expression was analyzed via real-time PCR. Statistical significance is indicated by an asterisk (*, $P < 0.05$, **, $P < 0.01$, and ***, $P< 0.001$).

# Discussion

Flavonoids are ubiquitous plant compounds that are present in physiologically significant quantities in our diet. [2] As such, it is important to better understand the benefits and consequences of its consumption. These compounds have been associated with modulation of cholesterol and inflammatory cytokine responses[31,32]. The data fully supports the latter claim. Quercetin and isorhamnetin strongly inhibited expression of IL-1β and IL-6 although this was not accomplished through AHR. There are other reported pathways that flavonoids can use to mediate expression of inflammatory cytokines , such as protein kinase C (PKC) and mitogen-activated protein kinase (MAPK).[36] Likewise, while flavonoids did not decrease cholesterol absorption via NPC1L1, it is entirely possible that the flavonoids mediate cholesterol homeostasis via the liver instead of the intestines. However, isorhamnetin did strongly inhibit expression of NPC1L1 mRNA. Furthermore, it is possible that the cell line does not produce sufficient

quantities of NPC1L1 protein in order to create a significant difference in cholesterol absorption. Thus, one cannot eliminate the possibility that NPC1L1 might be involved in isorhamnetin's mechanism of action.

In conclusion, flavonoids seem to be excellent anti-inflammatory agents, following the trends in the literature although AHR is not associated with its anti-inflammatory properties. While the data is inconclusive on the relationship between flavonoids and NPC1L1, isorhamnetin's ability to inhibit NPC1L1 mRNA suggests that flavonoids may maintain cholesterol homeostasis at least partially through modulation of NPC1L1 expression. More research should be done in order to properly determine whether flavonoids can moderate cholesterol homeostasis through NPC1L1.

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**Reading Hospital Internal Medicine Clinic** **Summer 2012**

## Publications:

Chua, C. O., Chahboune, H., Braun, A., Dummula, K., Chua, C. O., Yu, J., Ungvari, Z., Sherbany, A. A., Hyder, F., and Ballabh, P. (2009). Consequences of Intraventricular Hemorrhage in a Rabbit Pup Model. *Stroke* 40, 3369-3377.

## Activities:

**THON Finance Committee** **Fall 2010 – Spring 2012**