



HAL
open science

NF- κ B signaling as a putative target for omega-3 metabolites in the prevention of age-related macular degeneration (AMD)

Kai Kaarniranta, Antero Salminen

► **To cite this version:**

Kai Kaarniranta, Antero Salminen. NF- κ B signaling as a putative target for omega-3 metabolites in the prevention of age-related macular degeneration (AMD). *Experimental Gerontology*, 2009, 44 (11), pp.685. 10.1016/j.exger.2009.09.002 . hal-00536003

HAL Id: hal-00536003

<https://hal.science/hal-00536003>

Submitted on 15 Nov 2010

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Accepted Manuscript

NF- κ B signaling as a putative target for omega-3 metabolites in the prevention of age-related macular degeneration (AMD)

Kai Kaarniranta, Antero Salminen

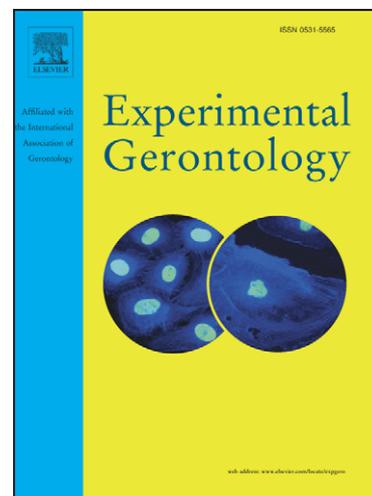
PII: S0531-5565(09)00171-5
DOI: [10.1016/j.exger.2009.09.002](https://doi.org/10.1016/j.exger.2009.09.002)
Reference: EXG 8645

To appear in: *Experimental Gerontology*

Received Date: 2 July 2009
Revised Date: 28 August 2009
Accepted Date: 8 September 2009

Please cite this article as: Kaarniranta, K., Salminen, A., NF- κ B signaling as a putative target for omega-3 metabolites in the prevention of age-related macular degeneration (AMD), *Experimental Gerontology* (2009), doi: [10.1016/j.exger.2009.09.002](https://doi.org/10.1016/j.exger.2009.09.002)

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.



NF- κ B signaling as a putative target for omega-3 metabolites

in the prevention of age-related macular degeneration (AMD)

Kai Kaarniranta^{a,b,*}, Antero Salminen^{c,d}

^aDepartment of Ophthalmology, Institute of Clinical Medicine, University of Kuopio,
P.O. Box 1627, FIN-70211 Kuopio, Finland

^bDepartment of Ophthalmology, University Hospital of Kuopio, P.O. Box 1777, FIN-
70211 Kuopio, Finland

^cDepartment of Neurology, Institute of Clinical Medicine, University of Kuopio, P.O.
Box 1627, FIN-70211 Kuopio, Finland

^dDepartment of Neurology, University Hospital of Kuopio, P.O. Box 1777, FIN-70211
Kuopio, Finland

*Corresponding author. Address: Institute of Clinical Medicine, University of Kuopio

P.O. Box 1627, FIN-70211 Kuopio, Finland, Tel., +358-17-172485, Fax: +358-17-

172486, Email: kaarnira@messi.uku.fi

Key Words: age-related macular degeneration, NF- κ B, omega fatty acids

ABSTRACT

Age-related macular degeneration (AMD) is the major reason of blindness of the elderly all over the world. AMD is characterized by a progressive loss of central vision attributable to degenerative and neovascular changes in the macula, the highly specialized region of the retina responsible for sharp and colour visual acuity. Degeneration and cell death of retinal pigment epithelial cells (RPE) cause secondarily adverse effects on neural retina leading to visual loss. Most AMD patients cannot benefit from any treatment modalities. The prevalence of AMD is rising as a consequence of the aging of the population. As the RPE cells age, they are subject to continued oxidative stress and this believed to induce inflammation and progression of AMD. Interestingly, many clinical trials have revealed that dietary intakes of omega-3 fatty acids can reduce the risk of both early and late AMD, although their molecular targets in cellular signaling in AMD pathology are not understood. Recently, it has been proposed that the omega-3 fatty-acid metabolites, resolvins and protectins, function as endogenous anti-inflammatory compounds. In this review, we propose that resolvins and protectins mediate their beneficial effects by preventing NF- κ B signalling and this that may represent a new target for regulating the inflammatory responses in AMD.

1. Age-related macular degeneration (AMD)

AMD is the leading cause of blindness of elderly people in the developed countries. According to World Health Organization (WHO), 50 million persons suffer AMD symptoms and 14 million persons are blind or severely visually impaired because of AMD (Gehrs et al., 2006). The disease affects the macula, which is located in the central area of the retina. AMD is characterized by degeneration of the macular retinal pigment epithelial (RPE) cells, Bruch's membrane, and choriocapillaris (Holz et al., 2004). Degeneration and cell death of RPE cells cause secondary adverse effects on neural retina leading to visual loss. AMD results in an inability to read, recognize faces, drive motor vehicles, or move freely. The AMD aetiology is known to be multifactorial, i.e. in addition to a strong genetic component, environmental risk factors such as aging, smoking, obesity, hypertension and hypercholesterolemia may predispose to AMD (Hyman and Neborsky 2002; van Leeuwen et al., 2003; Buch et al., 2005; Gehrs et al., 2006). At present, chronic oxidative stress and inflammation are strongly linked to AMD pathogenesis (Arjamaa et al., 2009; Kaarniranta and Salminen 2009). AMD is divided early and late diseases, as well as the atrophic and exudative degeneration categories (Holz et al., 2004). In the absence of effective treatment for AMD, the number of patients severely disabled by AMD is expected to increase in the next 20 years by more than 50%.

A hallmark of AMD is the detrimental accumulation of lysosomal lipofuscin in RPE and the presence of extracellular drusen deposits between the basal lamina of the RPE and the inner collagenous layer of Bruch's membrane (Fig. 1). A high amount of lipofuscin and drusens predict AMD advancing and severity. In response to chronic oxidative stress and

inflammation, choroidal neovascularization may be developed in certain AMD cases (15-20% of all). In the neovascularization process, new vessels sprout from the choroidal capillaries through Bruch's membrane into the sub-RPE space or into retinal layers (Fig.1). New treatments including laser therapy and anti-VEGF intravitreal injections for AMD are only useful in patients with exudative AMD (Schmidt-Erfurth and Prunte, 2007). The weak vessel structure mean that there may be leakage into the retinal layers, which evokes fibroglisis resulting in a disciform scar and severe visual loss if not treated appropriately.

One major public health challenge is to devise some effective primary prevention of AMD. To date, there are only recommendations for modifying the risk factors of AMD (Christen et al., 1996; Seddon et al., 1996). Interestingly, intake of dietary ω -3 fatty acids and/or fish, the main dietary source of long-chain ω -3 fatty acids, has been long claimed to be able to prevent AMD. In a recent meta analysis, Chong et al., submit the results of randomized controlled trials, prospective cohorts, case-control, and cross sectional studies, and came to the conclusion that consumption of ω -3 fatty acids may be associated with a lower risk of AMD (Chong et al., 2008).

ω -3 Fatty acids are essential fatty acids because humans cannot produce these components de novo and rely on diet as their sole source. Importantly, docosahexaenoic acid (DHA), a major representative of ω -3 fatty acids, is present in high concentrations in the retinal outer segments, and its deficiency may trigger the onset of AMD (Bazan 1989). There are several studies indicating that long-chain ω -3 fatty acids may also protect against oxidative stress, inflammation, and thus prevent age-related retinal damage (Ariel

and Serhan 2007; Hasturk et al., 2007; Schwab et al., 2007). Here, we discuss the role of ω -3 fatty acids and their metabolites in the regulation of oxidative stress and inflammation via NF- κ B signaling in RPE cells and AMD.

2. Putative role of ω -3 fatty acids in modulating factors and processes implicated in pathogenesis of AMD

Omega-3 fatty acids comprise of linolenic acid (a short-chain ω -fatty acid), docosahexaenoic acid (DHA, long-chain ω -3 fatty acids), and eicosapentaenoic acid (EPA, long-chain ω -3 fatty acids). Alpha-linolenic acid is the dietary precursor to both DHA and EPA and it can be converted to a long-chain ω -3 fatty acid (Ariel and Serhan 2007). DHA and EPA are the major representatives of the ω -3 fatty acids. Recently, advances have been made in understanding the potent actions of ω -3 fatty acids in controlling the resolution of inflammation (Ariel and Serhan 2007; Hasturk et al., 2007; Schwab et al., 2007).

Interestingly, very high levels of the DHA are present in the retina—specifically, in the outer segment disks of photoreceptor cells. The relative amount of DHA present of rod outer segment disks as compared to the amount of total fatty acids is the highest of any tissue (Stone et al., 1979). The high DHA content and its specific and consistent tissue distribution provide the basis for the belief that DHA has an essential functional role in the retina (Bazan 1989; SanGiovanni and Chew 2005; Bazan 2006). DHA influences the membranes' biophysical properties and is a central regulator of visual cycle (Gibson and Brown 1993). It also controls membrane transport systems and is a precursor in the

synthesis of other biologically active molecules. In view of the presence of such high levels of DHA in outer segment discs and its physiological roles, it could be anticipated that DHA may have a beneficial role in retinal degeneration diseases such as AMD.

EPA regulate lipoprotein metabolism and suppress the expression of various inflammatory compounds that can damage the extracellular matrix, including the Bruch's membrane, and lead to neovascularization of AMD (Holz 2004; Chong et al., 2008). Due to the crucial role of inflammation in the pathogenesis of AMD, it is conceivable that EPA might be a protective factor in preventing or at least slowing disease progression.

Recently, two novel families of compounds, resolvins and protectins, have been identified and these are believed to be involved in the resolution of inflammation. The resolvins are biosynthesized from the major ω -3 fatty acids EPA and DHA via cyclooxygenase-2 (Serhan 2007). The resolvins are local-acting mediators with potent anti-inflammatory properties including suppressing the production of pro-inflammatory mediators (Serhan 2007). In addition, they have the capacity to decrease the impacts of the induced immune response induced by reactive oxygen species. The protectins are produced and biosynthesized from DHA. One member of the protectin D family is neuroprotectin D1 (NPD1) that has antiapoptotic effects on the retinal pigment epithelial cells in response to oxidative stress (Mukherjee et al., 2004; Bazan 2005; Mukherjee et al., 2007). Deficiency in DHA associates with decreased resolvins and NPD1 levels that are estimated to elicit macular degeneration (Bazan 2009). Both families of lipid mediators, the resolvins and protectins, are potent local-acting activators of endogenous anti-inflammation mechanisms that promote the resolution of inflammation and prevent

oxidative stress –induced cellular damage such as occurs in the degenerative retinal disease – AMD (Fig. 1). In addition, ω -3 fatty acids have beneficial effects against obesity and insulin resistance that may be indication of systemic metabolic improvements (Robinson et al., 2007).

In recent years, the resolution of inflammation has emerged as an area with considerable therapeutic potential as there are known to be many local mediators that can be manipulated pharmacologically (Serhan 2007). Self-resolving inflammatory exudates revealed that the termination of acute inflammation involves active biosynthetic processes producing novel endogenous lipid mediators, such as resolvins and protectins, that may suppress inflammation. It is now clear that resolution of inflammation is an active rather than a passive process (Ariel and Serhan 2007). Although ω -3 fatty acids are in wide use as dietary supplements and potential therapeutic supplements in many diseases, including inflammatory diseases, their mechanisms and connection to inflammation signaling remain to be of elucidated.

3. NF- κ B as a target of ω -3 fatty acid metabolites in prevention of RPE degeneration

The NF- κ B system is a prominent mechanism responding to a variety of external and internal cellular danger signals, such as oxidative stress and immune activation (Salminen et al., 2008). The NF- κ B protein complex is retained in an inactive state in the cytoplasm by binding to inhibitory proteins such as I κ B α , I κ B β , I κ B γ , I κ B ϵ , and Bcl3 that are known as I κ B. If there is a stress stimulus, such as oxidative stress, I κ B kinase (IKK) is

activated which in turn phosphorylates I κ B proteins making them susceptible to ubiquitin-proteasome-system degradation (Barnes and Karin 2007). Subsequently, the NF- κ B complex undergoes nuclear translocation where it can bind to various promoter areas of its target genes and induce gene transcription of the corresponding genes that mostly are implicated in the regulation of inflammation. The NF- κ B family members comprise NF- κ B1 (p50/p105), NF- κ B2 (p52/p100), p65 (RelA), RelB and c-Rel proteins. The major NF- κ B regulatory complex is a heterodimer composed of a p50 and a p65 subunit that induces transcription of the corresponding genes e.g. TNF- α , IL-1 β , IL-2, IL-6, IL-8, IFN- γ , inducible NO synthase, adhesion molecules, cyclooxygenase-2 and acute phase proteins, such as CRP. In addition to the acute inflammation response, NF- κ B is a master regulator of chronic inflammatory processes (Barnes and Karin 2007; Salminen et al., 2008).

Recent studies have demonstrated that the ω -3 fatty acids inhibit the activity of the pivotal pro-inflammatory NF- κ B. Thus, ω -3 fatty acids via their metabolites resolvins and protectins can secondarily regulate the NF- κ B activity and thus the expression levels of various pro-inflammatory genes involved in acute and chronic inflammation (Ariel and Serhan 2007; Chung et al., 2009). The ω -3 fatty acids block the ligand-receptor interaction including TLR4 and TNF-receptors, prevent the IKK phosphorylation by several convergent pathways on the canonical NF- κ B pathway and attenuate the DNA-binding activity of NF- κ B transcription factor (Chung et al., 2009). All these inhibitory inputs of NF- κ B signaling could be postulated to suppress chronic inflammation in the retinal pigment epithelial cells and prevent the development of AMD.

Oxidative stress is a classical stress stimulus that can lead to inflammation in aged tissue such as occurs in AMD pathology (Fig. 1; Arjamaa et al., 2009; Kaarniranta and Salminen 2009; Chung et al., 2009). Chronic oxidative stress is known to induce the production of advanced glycation endproducts (AGE) and their receptors (RAGE). Interestingly, minimal immunolabeling for RAGE or AGE was found in RPE cell layers in normal retinas, whereas a clearly increased levels of both AGE and RAGE were identified in the RPE cells for samples isolated from AMD patients (Howes et al., 2004; Yamada et al., 2006). The increased AGE and RAGE strongly activated NF- κ B in cultured RPE cells and evoked apoptotic cell death (Howes et al., 2004). Interestingly recent observations reveal that ω -3 fatty acids attenuate choroidal neovascularization in mice and decrease the expression of several inflammatory markers e.g., intercellular adhesion molecule (ICAM) -1, monocyte chemotactic protein (MCP) -1, vascular endothelial growth factor (VEGF) and interleukin (IL) -6 (Koto et al., 2007). Furthermore, NF- κ B is a central regulator of IL-6, a cytokine that has been shown to be an important regulator of choroidal neovascularization (Fig. 1; Koto et al., 2007; Izumi-Nagai et al., 2007; Paimela et al., 2007; Salminen et al., 2008).

4. Conclusions

A new generation of lipid mediators, the metabolites of the ω -3 fatty acids, resolvins and protectins, provide real promise as endogenous anti-inflammatory agents. Plenty of studies indicate that regular dietary intakes of ω -3 fatty acids and fish are associated with a reduced risk of both early and late AMD. However, the pathogenetical mechanisms of their anti-inflammatory actions remain to be clarified. We propose that NF- κ B might be

an important target for these ω -3 fatty acid metabolites, resolvins and protectins in their abilities, to suppress oxidative stress induced inflammatory responses in RPE cells and in this way to retard AMD development. However, there is challenge to understand dosage and treatment schedules of omega-3 fatty acids in the prevention of multifactorial AMD.

References

- Ariel, A., Serhan, C.N., 2007. Resolvins and protectins in the termination program of acute inflammation. *Trends Immunol.* 228, 176-183.
- Arjamaa, O., Nikinmaa, M., Salminen, A., Kaarniranta, K., 2009. Regulatory role of HIF-1 α in the pathogenesis of age-related macular degeneration (AMD). *Ageing Res Rev.* 2009 Jul 7. [Epub ahead of print]
- Barnes, P.J., Karin, M., 1997. Nuclear factor-kappaB: a pivotal transcription factor in chronic inflammatory diseases. *N. Engl. J. Med.* 336, 1066-1071.
- Bazan, N.G., 1989. The metabolism of omega-3 polyunsaturated fatty acids in the eye: the possible role of docosahexaenoic acid and docosanoids in retinal physiology and ocular pathology. *Prog. Clin. Biol. Res.* 312, 95-112.
- Bazan, N.G., 2005. Neuroprotectin D1 (NPD1): a DHA-derived mediator that protects brain and retina against cell injury-induced oxidative stress. *Brain Pathol.* 15, 159–166.
- Bazan, N.G., 2006. Cell survival matters: docosahexaenoic acid signaling, neuroprotection and photoreceptors. *Trends Neuroscience* 29, 263-271.

Bazan NG. Cellular and molecular events mediated by docosahexaenoic acid-derived neuroprotectin D1 signaling in photoreceptor cell survival and brain protection. *Prostaglandins Leukot Essent Fatty Acids*. 2009 Jun 9. [Epub ahead of print]

Buch, H., Vinding, T., la Cour, M., Jensen, G.B., Prause, J.U., Nielsen, N.V., 2005. Risk factors for age-related maculopathy in a 14-year follow-up study: the Copenhagen City Eye Study. *Acta Ophthalmol.Scand.* 83, 409-418.

Chong, E.W., Kreis, A.J., Wong, T.Y., Simpson, J.A., Guymer, R.H., 2008. Dietary omega-3 fatty acid and fish intake in the primary prevention of age-related macular degeneration: a systematic review and meta-analysis. *Arch. Ophthalmol.* 126, 826-833.

Christen, W.G., Glynn, R.J., Manson, J.E., Ajani, U.A., Buring, J.E., 1996. A prospective study of cigarette smoking and risk of age-related macular degeneration in men. *JAMA* 276, 1147-1151.

Chung, H.Y., Cesari, M., Anton, S., Marzetti, E., Giovannini, S., Seo, A.Y., Carter, C., Yu, B.P., Leeuwenburgh, C., 2009. Molecular inflammation: Underpinnings of aging and age-related diseases. *Ageing Res. Rev.* 8, 18-30.

Gehrs, K.M., Anderson, D.H., Johnson, V., Hageman, G.S., 2006. Age-related macular degeneration--emerging pathogenetic and therapeutic concepts. *Ann. Med.* 38, 450-471.

Gibson, N.J., Brown, M.F., 1993. Lipid headgroup and acyl chain composition modulate the MI-MII equilibrium of rhodopsin in recombinant membranes, *Biochemistry* 32, 2438-2454.

Hasturk, H., Kantarci, A., Goguet-Surmenian, E., Blackwood, A., Andry, C., Serhan, C.N., Van Dyke, T.E., 2007. Resolvin E1 regulates inflammation at the cellular and tissue level and restores tissue homeostasis in vivo. *J. Immunol.* 179, 7021-7029.

Holz, F.G., Pauleikhoff, D., Spaide, R.F., Bird, A.C., 2004. Genetics of AMD. In: Holz FG, Pauleikhoff D, Spaide RF, Bird AC *Age-related Macular Degeneration*. Berlin, Heidelberg: Springer-Verlag 24–30.

Howes, K.A., Liu, Y., Dunaief, J.L., Milam, A., Frederick, J.M., Marks, A., Baehr, W., 2004. Receptor for advanced glycation end products and age-related macular degeneration. *Invest. Ophthalmol. Vis. Sci.* 45, 3713-20.

Hyman, L., Neborsky, R., 2002. Risk factors for age-related macular degeneration: an update. *Curr. Opin. Ophthalmol.* 13, 171-175.

Izumi-Nagai, K., Nagai, N., Ozawa, Y., Mihara, M., Ohsugi, Y., Kurihara, T., Koto, T., Satofuka, S., Inoue, M., Tsubota, K., Okano, H., Oike, Y., Ishida, S., 2007. Interleukin-6 receptor-mediated activation of signal transducer and activator of transcription-3 (STAT3) promotes choroidal neovascularization. *Am. J. Pathol.* 170, 2149-2158.

Kaarniranta, K., Salminen, A., 2009. Age-related macular degeneration: activation of innate immunity system via pattern recognition receptors. *J. Mol. Med.* 87, 117-123.

Kaarniranta, K., Salminen, A., Eskelinen, E.L., Kopitz, J., 2009. Heat shock proteins as gatekeepers of proteolytic pathways-Implications for age-related macular degeneration (AMD). *Ageing Res. Rev.* 8, 128-139.

Koto, T., Nagai, N., Mochimaru, H., Kurihara, T., Izumi-Nagai, K., Satofuka, S., Shinoda, H., Noda, K., Ozawa, Y., Inoue, M., Tsubota, K., Oike, Y., Ishida, S., 2007.

Eicosapentaenoic acid is anti-inflammatory in preventing choroidal neovascularization in mice. *Invest. Ophthalmol. Vis. Sci.* 48, 4328-4334.

van Leeuwen, R., Ikram, M.K., Vingerling, J.R., Wittteman, J.C., Hofman, A., de Jong, P.T., 2003. Blood pressure, atherosclerosis, and the incidence of age-related maculopathy: the Rotterdam Study. *Invest. Ophthalmol. Vis. Sci.* 44, 3771-3777.

Robinson, L.E., Buchholz, A.C., Mazurak, V.C., 2007. Inflammation, obesity, and fatty acid metabolism: influence of n-3 polyunsaturated fatty acids on factors contributing to metabolic syndrome. *Appl. Physiol. Nutr. Metab.* 32, 1008-1024.

Mukherjee, P.K., Marcheselli, V.L., Barreiro, S., Hu, J, Bok, D., Bazan, N.G., 2007. Neurotrophins enhance retinal pigment epithelial cell survival through neuroprotectin D1 signaling. *Proc. Natl. Acad. Sci. U.S.A.* 104, 13152–13157.

Mukherjee, P.K., Marcheselli, V.L., Serhan, C.N., Bazan, N.G., 2004. Neuroprotectin D1: a docosahexaenoic acid-derived docosatriene protects human retinal pigment epithelial cells from oxidative stress. *Proc. Natl. Acad. Sci. U.S.A.* 101, 8491–8496.

Paimela, T., Ryhänen, T., Mannermaa, E., Ojala, J., Kalesnykas, G., Salminen, A., Kaarniranta, K., 2007. The effect of 17beta-estradiol on IL-6 secretion and NF-kappaB DNA-binding activity in human retinal pigment epithelial cells. *Immunol. Lett.* 110, 139-144.

Salminen, A., Ojala, J., Huuskonen, J., Kauppinen, A., Suuronen, T., Kaarniranta, K., 2008. Interaction of aging-associated signaling cascades: inhibition of NF-kappaB signaling by longevity factors FoxOs and SIRT1. *Cell. Mol. Life Sci.* 65, 1049-1058.

SanGiovanni, J.P., Chew, E.Y., 2005. The role of omega-3 long-chain polyunsaturated fatty acids in health and disease of the retina. *Prog. Retin. Eye Res.* 24, 87-138.

Schmidt-Erfurth, U.M., Prunte, C.M., 2007. Management of neovascular age-related macular degeneration. *Prog. Retin. Eye Res.* 26, 437-451.

Schwab, J.M., Chiang, N., Arita, M., Serhan, C.N., 2007. Resolvin E1 and protectin D1 activate inflammation-resolution programmes. *Nature* 447, 869-874.

Serhan, C.N., 2007 Resolution phase of inflammation: novel endogenous anti-inflammatory and proresolving lipid mediators and pathways. *Annu. Rev. Immunol.* 25, 101-137.

Serhan, C.N., Chiang, N., Van Dyke, T.E., 2008. Resolving inflammation: dual anti-inflammatory and pro-resolution lipid mediators. *Nat. Rev. Immunol.* 8, 349-361.

Stone, W.L., Farnsworth, C.C., Dratz, E.A., 1979. A reinvestigation of the fatty acid content of bovine, rat and frog retinal rod outer segments. *Exp. Eye Res.* 28, 387-397.

Yamada, Y., Ishibashi, K., Ishibashi, K., Bhutto, I.A., Tian, J., Luty, G.A., Handa, J.T., 2006. The expression of advanced glycation endproduct receptors in RPE cells associated with basal deposits in human maculas. *Exp. Eye Res.* 82, 840-848.

Figure Legends

Fig. 1. Schematic presentation of AMD pathology and NF- κ B suppression with resolvins and protectins that might lead to resolution of inflammation in RPE cells and prevent AMD progression. Abbreviations: BM, Bruch's membrane; NF- κ B, nuclear factor κ B; VEGF, vascular endothelial growth factor.

ACCEPTED MANUSCRIPT

Fig.1

