Curative and preventive treatment for cardiovascular disease (CVD)

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BULLET POINTS

- Chronic diseases, including CVD, have multifactorial pharmacology (polypharmacology) and matching multifactorial drug formulation for effective treatment.
- Constructed a pathophysiological overview of CVD (Thromboembolic cascade) and identified 3 druggable targets, including maintenance of the integrity and health of the cell's protective cell coat (glycocalyx) and mitigate excessive oxidative and inflammatory damages.
- Synthesized 8 new chemical entities (NCEs) and formulated twelve different 3-NCE combos
- Designed the Tunac Arterial Plaque (TAP) mouse model to mimic the natural progression of CVD in humans. Histopathology of plaques showed well defined fibrous caps, which mimic humans
- Identified 4 blood markers and proved to reflect disruption of the glycocalyx, namely: Heparan sulfate (HS), hyaluronan synthase (HAS), syndecan-1 (SDC-1), and plasminogen activator inhibitor-1 (PAI-1)
- Tested the twelve 3-NCE combos vs the TAP model and identified 3 promising leads that reverse and cure plaques, namely Combos F, J and K. Combo K (3 NCE combo of FTX-214, -218, -219) is chosen as the lead candidate and hereby designated as *Embotricin*™
- Histopathology proved the effectiveness of the 4 blood markers as a diagnostic for plaque formation
- In summary: proof-of-principle preclinical of the effectiveness of 3-combo therapy vs CVD and accompanying diagnostics to follow plaque formation

INTRODUCTION

1. INCIDENCE OF CARDIOVASCULAR DISEASE (CVD)

CVD is the leading cause of death and disability, killing 655,000/year in the US (CDC) and 17.9 million people/year in the world (WHO), which accounts a third of all deaths on the planet and half of all non-communicable-disease-related deaths. The available drugs vs CVD are symptom targeted, which are palliative at best. There is no curative drug against this disease because the pervading sense of obsession on cholesterol-lowering paradigm has discouraged alternative drug development *(Fig.1).*

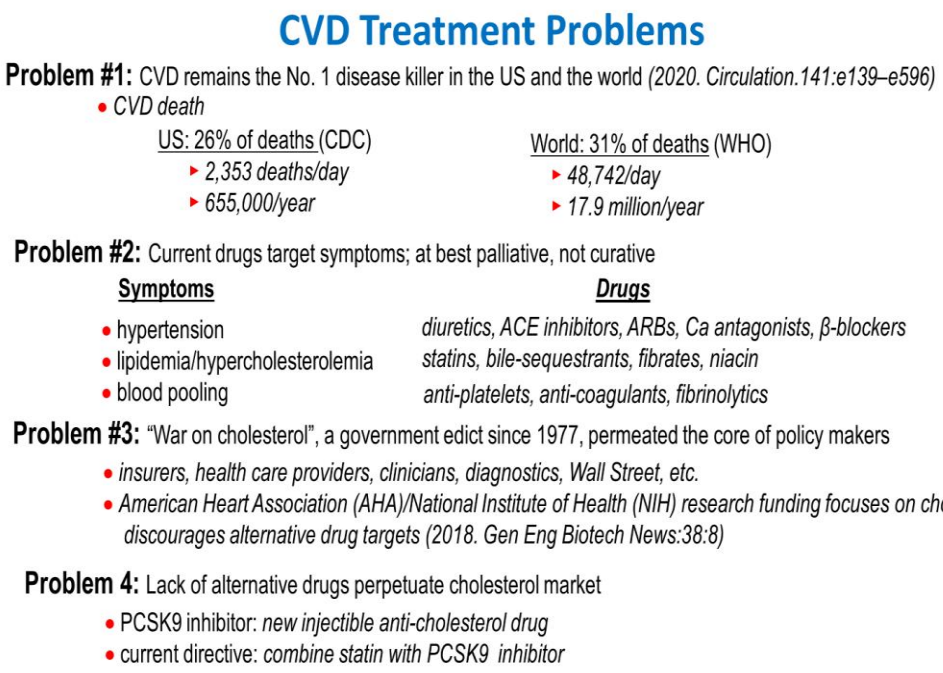

Figure 1. Cardiovascular disease (CVD) as number 1 killer and associated problems for developing a cure

This paper reports on the discovery and development of a series of 3-NCE components found curative and preventive of atherosclerotic plaques in preclinical setting, targeting the polypharmacology nature of the disease.

2. THE MULTIFACTORIAL CAUSES OF CVD

2.1. Dysfunctional blood flow: The cardiovascular system consists of the heart, which pumps blood through branching arteries, arterioles and capillaries and used blood is returned through the veins per muscle contraction squeezing blood back to the lung for re-oxygenation. A balanced blood pressure and osmotic pressure create a healthy blood flow, which is aided by a slippery lining at the surface of the vessel called glycocalyx (GCX). GCX allows red blood cells (RBC) to glide through the narrow capillary beds *(Fig. 2)*

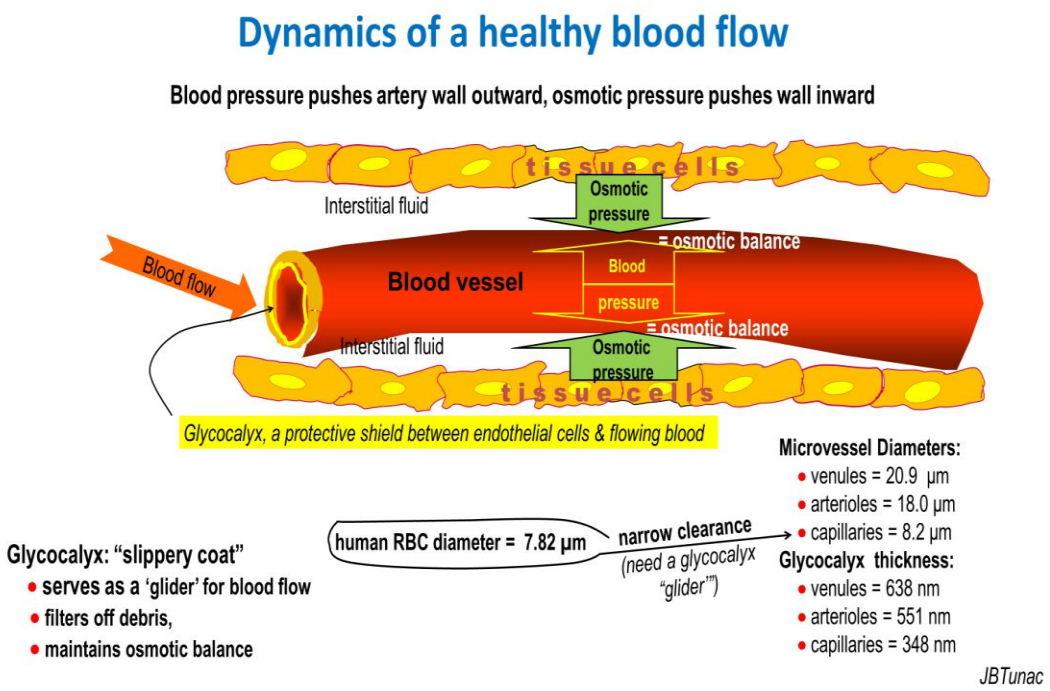

Figure 2. Blood vessel is lined with a 'slippery coat' called glycocalyx that promote healthy blood flow dynamics.

2.2. Disruption of Glycocalyx (GCX) affects blood flow. All cells in the human body are covered by GCX, which provides the first line of protection from physical, chemical, and biological wear and tear. When GCX covers the surface endothelium of arteries, it is called arterial endothelial glycocalyx (AEG). AEG serves as a protective lining, filters off cell debris, nest to blood-flow regulating components and as a sensing medium. AEG senses changes in the microenvironment and accordingly regulate vascular tone, circulating cell adhesion, coagulation, fibrinolysis, and vessel wall inflammation. Disruption of AEG is a first step in the atherosclerosis process, which is reflected by shedding of components including proteoglycans (GAG chain attached to protein) and glycoproteins (sugars attached to protein) and serves as a nest to blood-flow regulating components (2016.Atherosclerosis. 252:136–146). The AEG is a complex of proteoglycans (GAG chain attached to protein) and glycoproteins (sugars attached to protein) ***(Fig 3).***

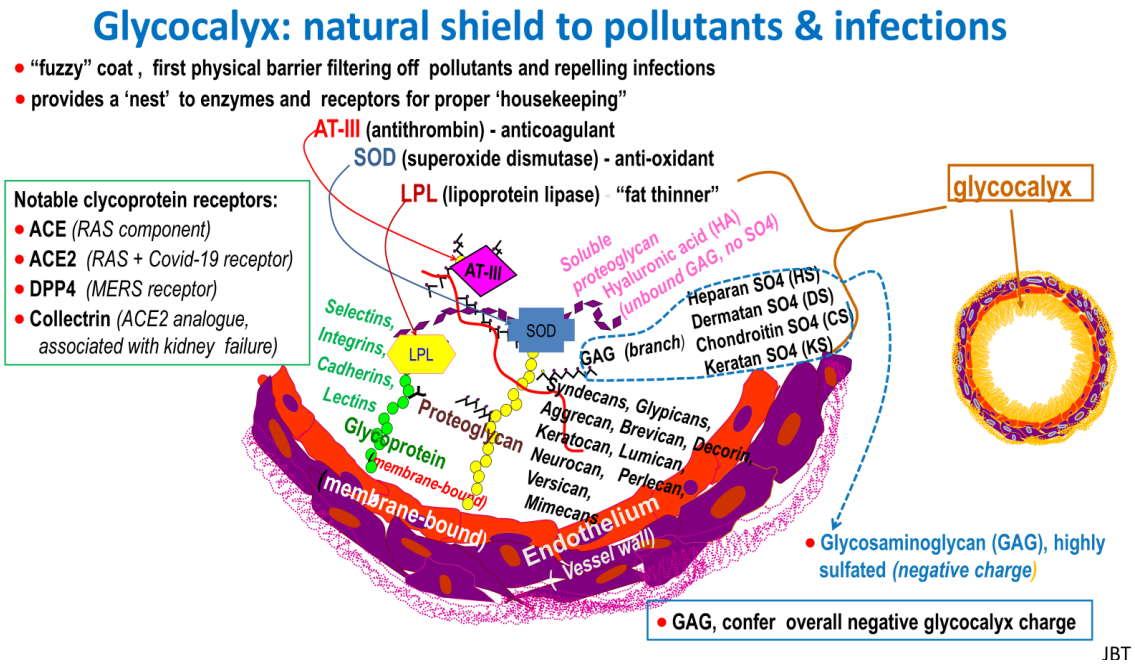

Figure 3. Anatomy of the protective endothelial glycocalyx showing glycoprotein and proteoglycan components including nested proteins (AT-III, SOD, LPL)

2.3. Environmental pollutants and diet produce oxidants. Reactive oxygen species (ROS) are highly reactive chemical molecules formed due to the electron acceptability of O_2. Oxygen has two unpaired electrons, which makes it especially susceptible to radical formation. Thus, sequential reduction (addition of electrons) of molecular oxygen leads to formation of a group of ROS, which is the bane to all aerobic species. All ROS are extremely harmful to organisms at high concentrations that exceeds the defense mechanism. Excess ROS 'steals' electron from nearby atoms creating a 'foreign or antigenic' molecule and a cell under oxidative stress *(Fig.4)*

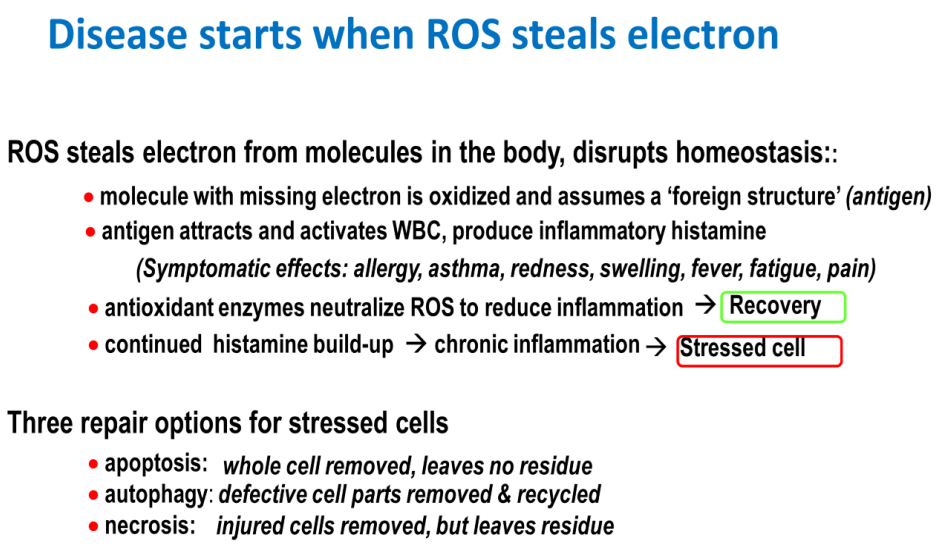

Figure 4. Cells containing molecules with missing electron (oxidized) is under oxidative stress and if not promptly repaired create various diseases.

Intrinsic ROS is produced as a by-product in the oxidation of food. The right quantity and food quality produce beneficial levels of ROS, which is maintained by built-in antioxidant enzymes. However overeating and high fat diet plus exposure to stressful environment produce extraneous ROS, which are oxidative to cells *(Fig. 5)*

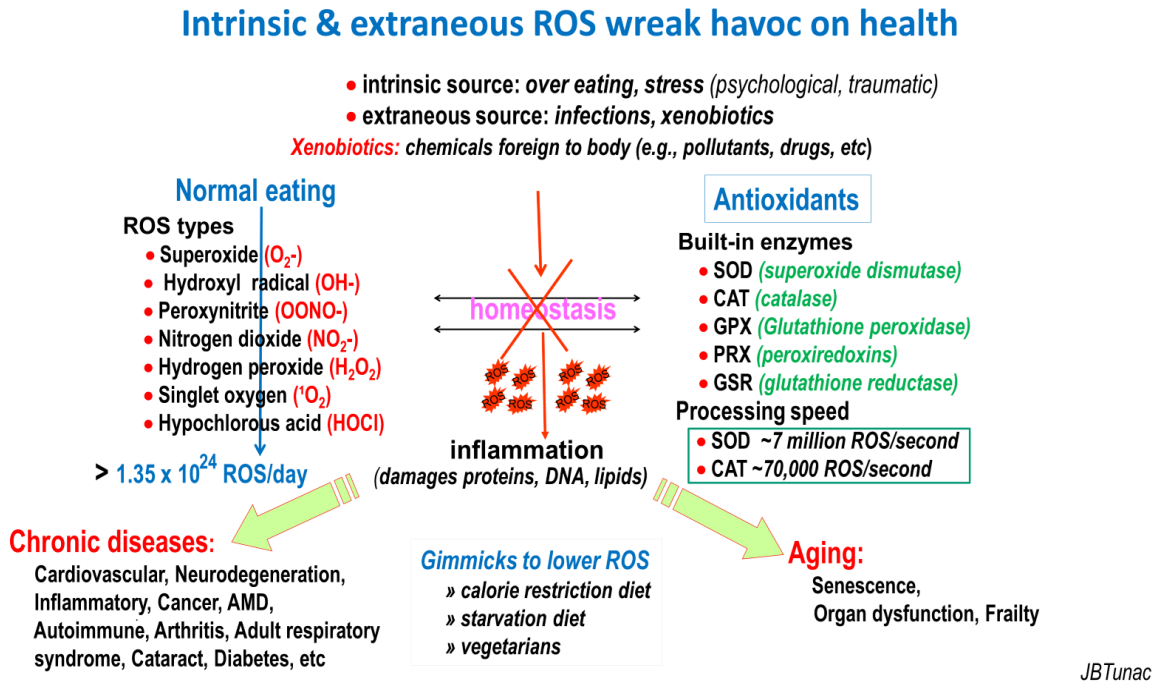

Figure. 5. Excess reactive oxygen species (ROS) steal electrons, oxidize cellular molecules and trigger diseases and aging

Along with high fat diet, xenobiotics or environmental pollutants are the main risk factors for CVD. Environmental pollutants oxidize the protective coating of the cell (2017. Lancet 389:1907-18) and damaged or oxidized components cells become antigenic triggering inflammation and additional ROS, which cascades into diseases *(Fig 6).*

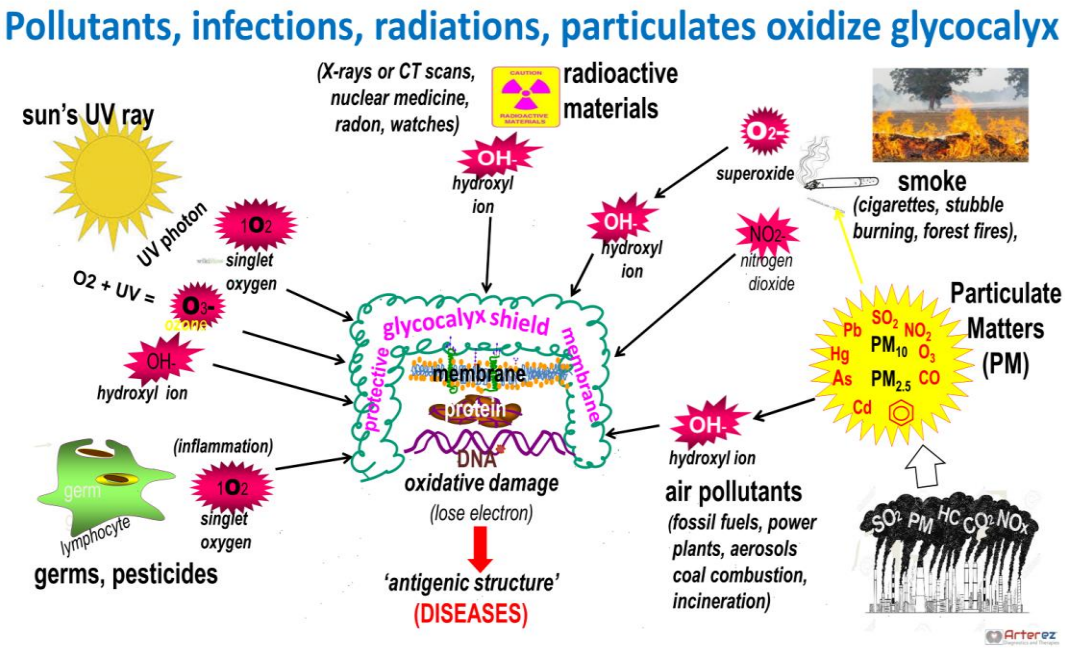

Figure 6. The environment factors including pollutants, infections, ozones and chemicals packaged in particulate matters (PMs) stress or disrupt cells

3. DRUG SOURCE AND DISCOVERY

3.1. Folk medicines. First medicines came from natural products (folk medicine), which were a mixture of compounds, Subsequently, advances in chemistry allowed the purification of each of the component compounds and the most active identified. For example, the active component, morphine, was isolated from opium; quinine (malaria treatment) from cinchona bark; colchicine (gout treatment) from autumn crocus; atropine from Atropa belladonna; and, cocaine (local anesthetic) was from coca leaves. These led to the adoption of the "one drug-one target-one disease" philosophy that focused on one active component to a specific disease or biological target, which became the current paradigm of drug discovery. This paradigm evolved the in vitro high through-put screening (HTS) matching one compound to one disease indication or target, which accounted for the discovery of numerous antibiotics and chemotherapies and marked an unprecedented progress in therapeutics during the decades after World War II (1999. Rise and Fall of Modern Medicine Little Brown & Co). Moreover, synthesis of active analogs catered the development of computer-based chemistry including structure–activity relationship (SAR) and computer-aided drug (CAD) development (2005. Drug Discov. Today. 10:895–907). SAR and CAD prompted the synthesis of numerous analogs of penicillins, carbapenems, cephalosporins, azithromycins, etc (2012. J Antibiotics 65:385–395). However, mass screening of thousand compounds per day ultimately became inefficient (2010. Curr. Pharm. Biotechnol.11:764–778)

because in vitro activity does not necessarily translate to in vivo or animal activity (2015. Nat. Rev. Drug Discov. 14: 475-486). The "one drug-one target-one disease" philosophy 'is blind' to the complex nature of biological systems (2011. J. Comput. Aided Mol. Des. 25:699–708).

3.2. Cholesterol as a "one-drug-one target" for treatment of CVD.

In keeping with the "one-drug-one target" philosophy, Akira Endo of Japan (Sankyo) identified the enzyme 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG CoA) reductase as a drug target for antifungal antibiotic (fungal membrane being ergosterol synthesized via HMG CoA route and inhibiting this route kills molds). In vitro mass screening of microbial extracts led to the discovery of citrinin and compactin (aka: mevastatin, mevinolin), the first statin (1976. J Antibiot. 29:1346-8). However, these compounds were not developed as antifungals; instead, evaluated to lower serum cholesterol (1974. Proc Natl Acad Sci USA 71:788–92) but failed in rats and mice (2004. Atherosclerosis Supplements 5: 13–16). Also, Merck discovered lovastatin (aka, mevinolin, monacolin K) a compactin analog that inhibits HMGCoA: both compactin and lovastatin were dropped because of toxicity or clinically ineffective ()

In 1961, the American Heart Association (AHA) championed the "Cholesterol Hypothesis" limiting egg consumption to reduce cardiovascular disease (CVD). AHA lobbied the government to declare" War on Cholesterol" resulting in the funding of cholesterol-related research including familial hypercholesterolemia (FH). Soon, Michael Brown and Joseph Goldstein (University of Texas Health Science Center) identified a defective low-density lipoprotein (LDL) receptor (LDL-R) gene responsible for blocking the absorption of LDL-cholesterol (1974. J. Biol Chm 249: 5153-62).

> *Cholesterol is always packaged in lipoproteins; there is no free-floating cholesterol in the blood stream or free cholesterol sticking onto arterial wall. Since cholesterol is insoluble in water, it is specially packaged along with fats for delivery to the blood stream in lipoproteins, including chylomicron, very low density lipoprotein (VLDL), intermediate density lipoprotein (IDL), low density lipoprotein (LDL) and high density lipoprotein (HDL). Apoprotein is the protein in lipoproteins that dictates their corresponding binding site: for example, apo B in LDL binds to the cell's LDL-R receptor, while apo E in VLDL, IDL, HDL binds with plasma protein for blood transport. VLDL packages cholesterol synthesized in the liver (along with dietary fats) for delivery into the bloodstream; once VLDL unloads its fat cargo, it becomes IDL; subsequently, IDL unloads its fat cargo to become LDL, and unloading of fat from LDL becomes HDL. LDL is the specific lipoprotein that delivers functional cholesterol to the cells: the protein portion of LDL binds to the cell's LDL receptor (LDL-R) and once bound cholesterol is delivered via endocytosis.*

A defective LDL-R results in a buildup of LDL on arterial surface and a sequelae of FH hypercholesterolemia, which overtime develops as cholesterol plaques. Hence, the association of hypercholesterolemia with atherosclerosis and the rationale to reduce LDL-cholesterol to treat atherosclerosis and the moniker that LDL is a "bad cholesterol". For this reason, Brown and Goldstein tested Endo's compactin, which effectively decreased LDL in cell culture (1978. J Biol Chem 253:1121–8) and in clinic (1980. Atherosclerosis 35:259–66); Endo declined further clinical development because of tumorgenecity concerns. Alternatively, Brown and Goldstein turned to Merck and urged Merck to re-evaluate the failed lovastatin vs patients with severe FH (2004. Atherosclerosis Supplements 5: 13–161). Lovastatin was clinically effective and approved by FDA to treat FH; subsequently, lovastatin (Mevacor®) was prescribed to the general public to reduce cholesterol and treat CVD (*Fig. 7)*

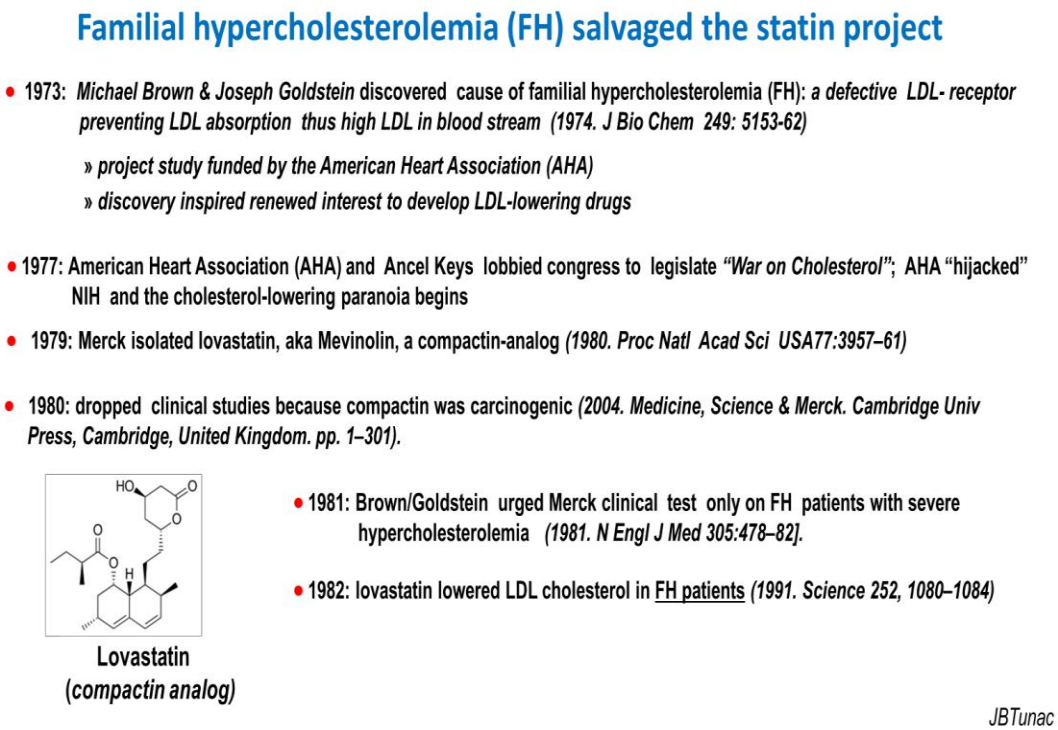

Figure 7. Lovastatin lowered plasma cholesterol in patients with familial hypercholesterolemia (FH).

3.3. Cholesterol metabolism

The classic knowledge on cholesterol metabolism was borne out of an experiment by a Russian scientist (Ignatowski) who observed arterial lesions (aka, "fatty streaks", "cholesterol plaque") in rabbits fed with meat, eggs, and milk. This was expected because a rabbit is an herbivore and cannot metabolize animal-based diet; such arterial lesions in regressed when switched to regular chow (1976. Ann N Y Acad Sci 275(1):363–78.). Regardless, the rabbit became a de facto model in the study of the pathogenesis and development of human atherosclerosis *(Fig. 8).*

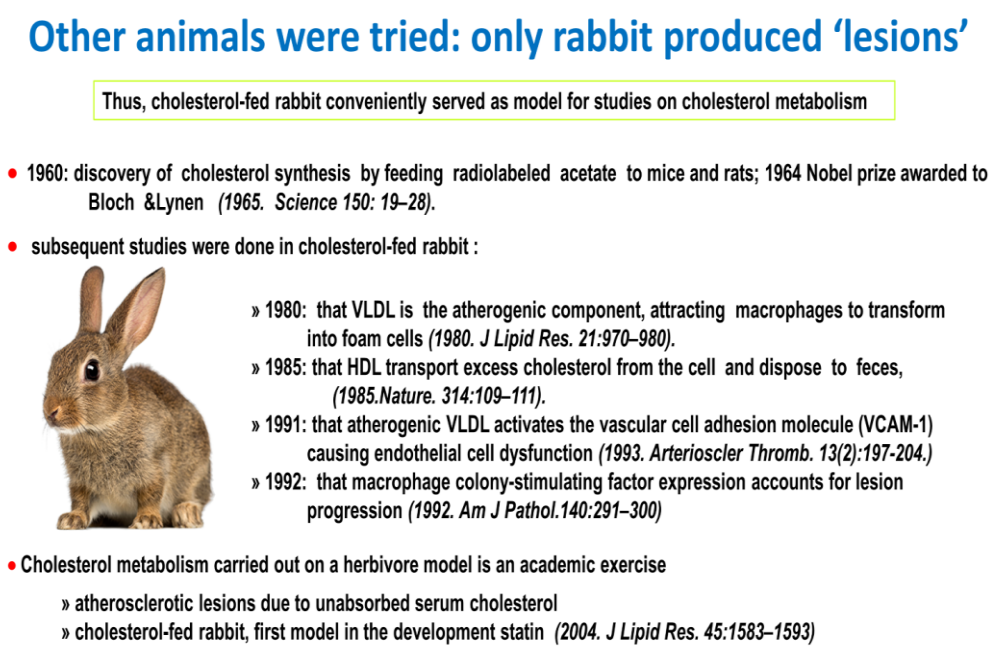

Figure 8. The rabbit as the central animal model in studying cholesterol biosynthesis.

4. ANIMAL MODELS FOR DRUG DISCOVERY

4.1. Gene deficient animals as alternative models for atherosclerosis.

The discovery of the LDL-R gene inspired the development of KO mice as models of cholesterol metabolism, including the LDL-R (*LdlR*-/-) mouse (1993. J Clin Invest 92:883–93) and the apolipoprotein E (*ApoE*-/-) mouse (1992. Cell 71:343–53; 1992. Science 258:468–71). Mice have a shorter time frame than rabbit, lower breeding cost and KO mice are suitable for high-throughput screening increasing the rate of discovery. In an ApoE−/− mouse, VLDL, IDL, HDL are not absorbed and thus stay in the blood stream and overtime "stick" on the arterial wall. Currently, the ApoE−/− mouse is the model of choice in the study of human atherosclerosis and accounted for the development of more statins including the new PCSK9 inhibitors Repatha and Praluent (*Fig. 9).*

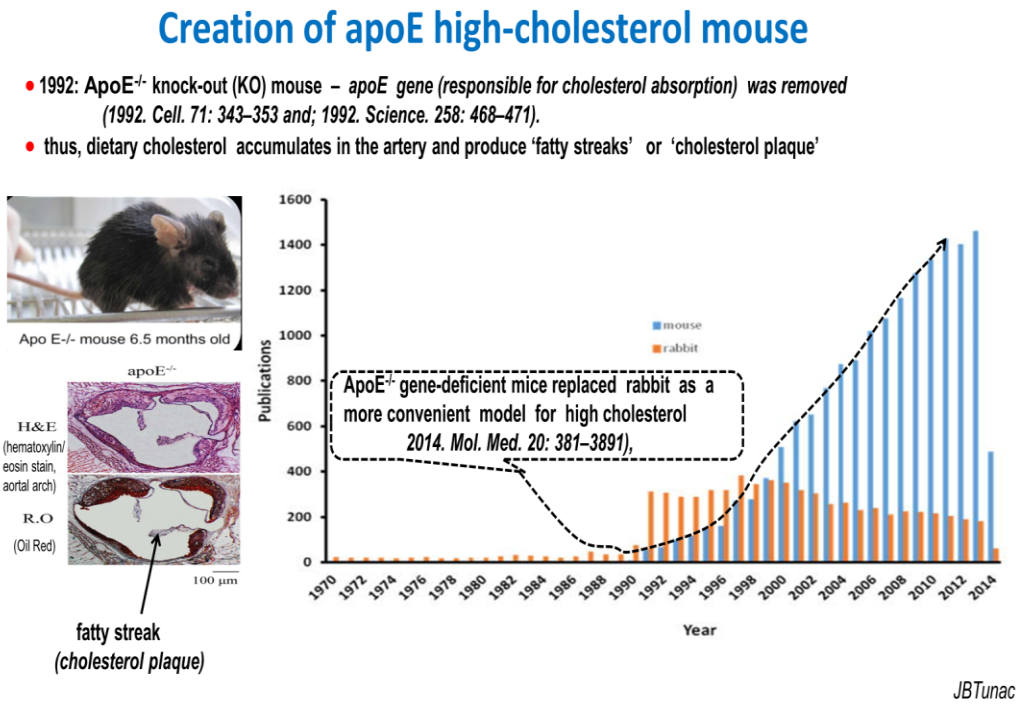

Figure 9. Historical use of rabbit and ApoE$^{-/-}$ mouse as models of atherosclerosis.

4.2. Classification of atherosclerotic plaques

Traditionally, two types of clinical plaques are described based on rabbit model: fatty streak (a thin lipid deposit in thin intima in children) and fibrous plaque (a thick fibrolipidic lesion in adults). Fatty streaks in infants was described as yellow dots, visible to the unaided eye at the root of the aorta, become more extensive at puberty (fibrous plaque) and in adults, develop to complex fibrolipid lesions called plaques (1957. Am J Pathol.1957; 33:875-885). The World Health Organization ((WHO Tech Rep Ser.1958; 143:1-20) classified plaques as atheroma (predominantly lipid component) and fibrous (predominantly collagenous component). Other terms include fibroatheroma, atheromatous plaque, fibrolipid plaque, or fibrofatty plaque to mean atherosclerosis (1986. Colour Atlas of Cardiovascular Pathology. London, England: Harvey Miller Publishers. 73)

On the other hand, AHA initially, classified plaques into 3 types; the assumption was for type I to progress through type III (Fig 24). However, the transition from type II to type III was not well defined, and thus the use of ApoE−/− knock-out mouse to model plaque transition and classification *(Fig. 10).*

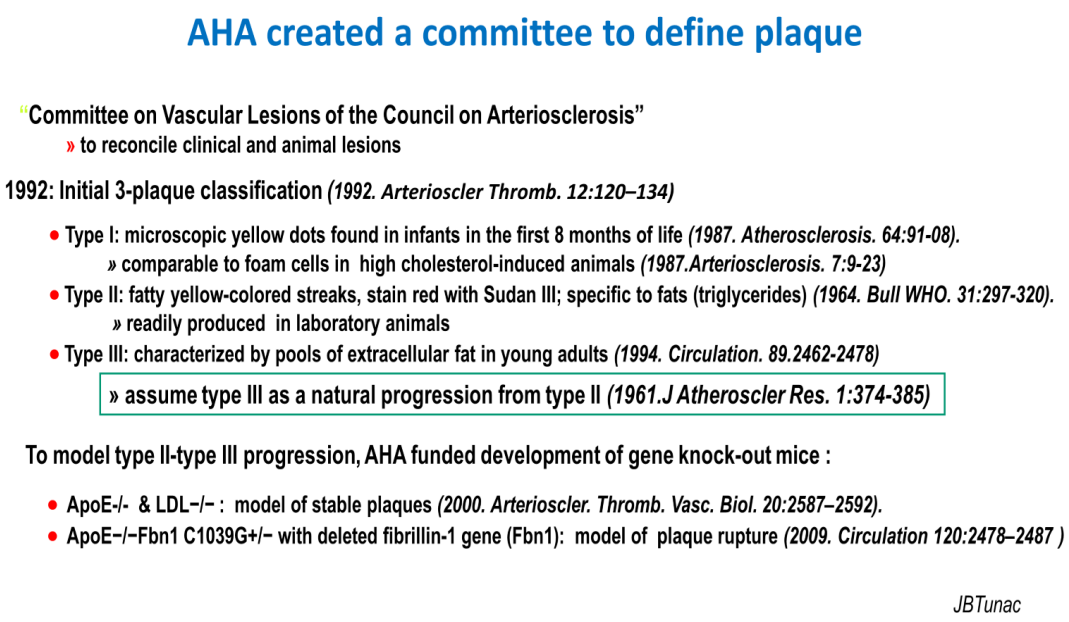

Figure 10. Three types of plaques in an attempt to unify rabbit lesions with clinical data.

4.3. KO mouse or rabbit plaques not the same as humans

Although KO mice are useful in understanding the concepts of plaque rupture, none of them exhibit the full combination of the characteristics seen in ruptured human plaques. Plaque rupture with occlusive thrombus, which is not modeled in KO mice (2010. Curr. Opin. Lipidol., 21: 434-440); clinical events such as MI or ischemic stroke are almost never seen in these models (2011. Thromb. Haemost., 106: 1-19). KO mice do not reflect the human form of atherosclerosis (2011. Nature. 473: 317–3251) and thrombosis (2010. Curr. Opin. Lipidol., 21: 434-440). The use of KO mice as model are generally flawed because "cholesterol plaques" are superficial with no fibrous cap but a "glue" of elastin-rich thin lamellae (2007. Arterioscler, Thromb Vasc Biol. 27:705–713; 2013.J Biomech.46: 716–722; 2008. Curr Drug Targets. 9:210–216), while human plaques are subendothelial with thick fibrous cap of collagen and elastin (2006. Arterioscler Thromb Vasc Biol. 26: 319–325), *Fig. 11.*

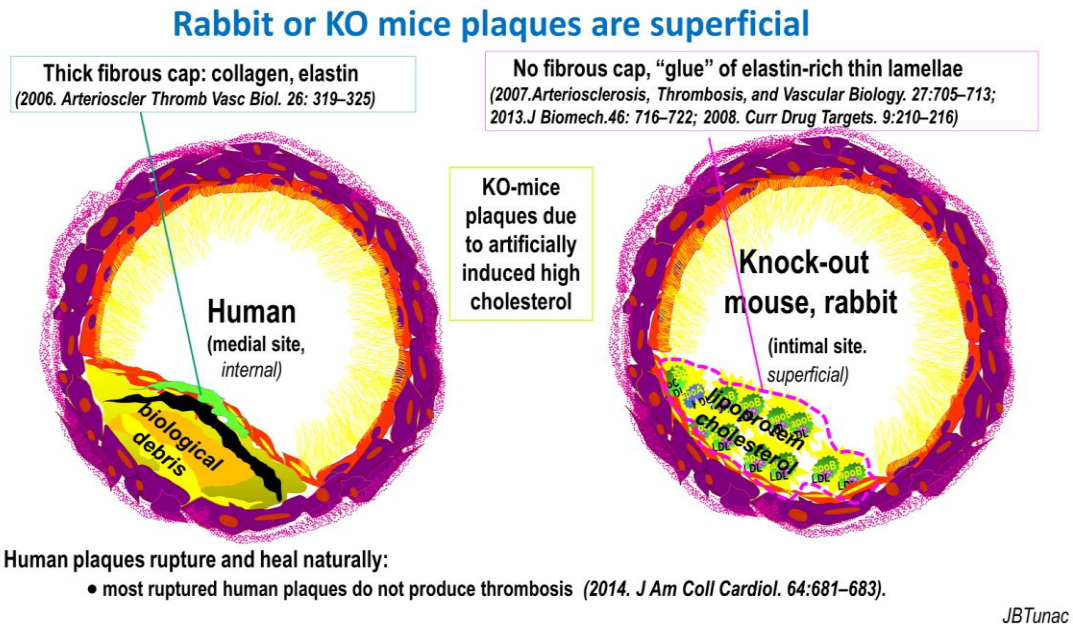

Figure 11. Plaques produced in animal models are superficial, while human plaques are subendothelial

OBJECTIVES

Our previous study showed the development of wild mouse to model atherosclerosis ((2017. J Clin Exp Cardiolog Suppl 8:1). Briefly, arterial plaque formation was achieved by treating wild c57Bl/6 with high fat and PCB produced plaques per histopathology. The objectives of this study are:

1. rationally synthesize a series of chemicals targeting specific sites in the thromboembolic cascade.
2. test these chemicals in combo to capture the polypharmacologic nature of chronic diseases.
3. correlate previously chosen blood markers per ELISA tests with histopathology targeting plaque as endpoint to drug effectiveness
4. evaluate drug effectiveness in both preventive and curative mode

MATERIALS AND METHODS

1. Mice

KO and inbred mice offer specific targets involving cholesterol metabolism but do not reflect the polypharmacological nature of complex diseases. Most importantly, there is no animal model that represents glycocalyx disruption in relation to CVD. To model the natural the genesis of CVD close to humans we developed a natural mouse model, the Tunac Arterial Plaque (TAP) mouse™ (2017. J Clin Exp Cardiolog Suppl 8:1). Briefly, this involves feeding mice with high fat diet and exposure to biological and chemical agent PCB. Thus, eighty-four (84) ten-week-old male C57/Bl6 mice were obtained from Jackson Laboratories. Three mice were raised from 6 weeks on a regular diet and served as controls, and the remaining mice were raised on a 60% fat diet (D12451, DIO series diet, Opensource Diets).

2. Treatments

Persistent environmental pollutants including the lipophilic persistent organic pollutants (POPs) polychlorobiphenyls (PCBs) bio-accumulate in fatty tissues (2009. Environ Health Perspect.117:417–25). PCB was chosen for this study because they are the most ubiquitous environmental pollutant. PCBs are resistant to acids and bases as well as to heat, and have been used as an insulating material in electric equipment, such as transformers and capacitors, and also in heat transfer fluids and in lubricants, as plasticizers, surface coatings, inks, adhesives, flame-retardants, paints, and carbonless duplicating paper. Pollutants disrupt certain signaling and differentiation pathways and to induce inflammation in the adipose tissue (2007. Chemosphere 67:1463-7). For this study we evaluated 3,3',4,4'-Tetrachlorobiphenyl (PCB-77), which was obtained from Neosyn Laboratories. The dry chemical was suspended in 15.22 ml of corn oil to deliver 200 µmol/kg in 0.2 ml by gavage per mouse according to the treatment schedule A series of 12 combo compounds labeled A-L were tested. These combo compounds were suspended in 8 ml carboxymethylcellulose and 0.2 ml/mouse was delivered by gavage according to the treatment schedule *(Table 1).*

TABLE 1. Experimental setup showing treatment schedule to reflect preventive and curative modes. DIO (high-fat diet); A-L (3-combo compounds); PCB (chemical pollutant); sac (harvest)

	Group	Diet	Day 1	2	3	4	5	6	7	8	9	11	18
Preventative	1	DIO	A	A	A	A	A		PCB		PCB		Sac
	2	DIO	B	B	B	B	B		PCB		PCB		Sac
	3	DIO	C	C	C	C	C		PCB		PCB		Sac
	4	DIO	D	D	D	D	D		PCB		PCB		Sac
	5	DIO	E	E	E	E	E		PCB		PCB		Sac
	6	DIO	F	F	F	F	F		PCB		PCB		Sac
	7	DIO	G	G	G	G	G		PCB		PCB		Sac
	8	DIO	H	H	H	H	H		PCB		PCB		Sac
	9	DIO	I	I	I	I	I		PCB		PCB		Sac
	10	DIO	J	J	J	J	J		PCB		PCB		Sac
	11	DIO	K	K	K	K	K		PCB		PCB		Sac
	12	DIO	L	L	L	L	L		PCB		PCB		Sac
Control	13	DIO							PCB		PCB	Sac.	
Curative	14	DIO	PCB		PCB		A	A	A	A	A		Sac
	15	DIO	PCB		PCB		B	B	B	B	B		Sac
	16	DIO	PCB		PCB		C	C	C	C	C		Sac
	17	DIO	PCB		PCB		D	D	D	D	D		Sac
	18	DIO	PCB		PCB		E	E	E	E	E		Sac
	19	DIO	PCB		PCB		F	F	F	F	F		Sac
	20	DIO	PCB		PCB		G	G	G	G	G		Sac
	21	DIO	PCB		PCB		H	H	H	H	H		Sac
	22	DIO	PCB		PCB		I	I	I	I	I		Sac
	23	DIO	PCB		PCB		J	J	J	J	J		Sac

	24	DIO	PCB		PCB		K	K	K	K	K		Sac
	25	DIO	PCB		PCB		L	L	L	L	L		Sac
	26	DIO	PCB		PCB	Sac							
	27	DIO											Sac
Control	28	5001											Sac

3. Enzyme-linked immunosorbent assay (ELISA)

A blood tests to detect antigens, in this case disrupted or glycocalyx debris. Four test kits were used to analyze the collected plasma samples: Heparan Sulfate ELISA and Hyaluronan Synthase 1 (HAS-1) ELISA (Antibodies-Online), Total Plasminogen Activation Inhibitor-1 (PAI-1) ELISA (Molecular-Innovive) and Syndecan 1 (SDC1) ELISA (USCN, Houston, Texas). All tests were performed on plasma, diluted to the fall within the standard curve if necessary, and carried out according to the manufacturer's instructions.

4. Sacrifice and Harvest

The mice were sacrificed on days 4, 11, or 18 according to the experimental plan (three each from groups). The animals were anesthetized by intraperitoneal injection of 90 mg/kg Ketamine and 8 mg/kg Xylazine, and Isoflurane gas anesthesia. Blood was collected by retro-orbital bleeding or from the heart and mixed with 50 mg/ml heparin to prevent clotting. The thorax was opened to expose the heart, and saline was injected into the left ventricle, with the right atrium opened to allow the drainage of blood and saline. The heart was perfused with at least 5 ml of saline and until no blood was observed in the drainage from the atrium. The heart was carefully dissected and frozen for histological sectioning. Plasma was collected from the blood samples by centrifuging at 1000 rpm for 15 minutes and collecting the supernatant. The samples were stored at -80°C until analysis.

RESULTS

1. Diseases and polypharmacology. Complex diseases require complex therapeutic approaches that "hits" multiple targets, which is the platform of polypharmacology. Thus, the current challenge the multiple targets (multiple associated with polypharmacology (2010. Drug Discov. Today. 15:749–756). In our quest to design curative therapy vs CVD, we sketched cascade, which we believe reflects the complex thromboembolic pathway and identified druggable targets *(Fig. 12)*

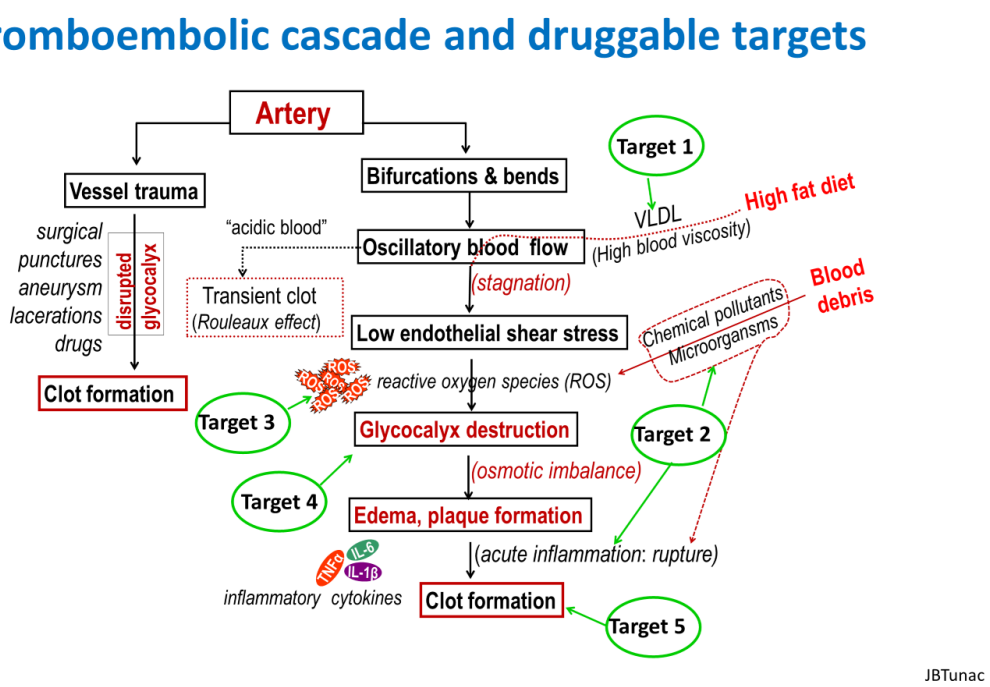

Figure 12. The thromboembolic cascade and identified 'druggable' targets

2. Synthesis of polypharmacology components.
Based on experience in drug development, we identified naturally occurring substances found in the body (to mitigate toxicity) with therapeutic activities and used them as building blocks for new chemical entity (NCE) synthesis, including:

- Indole - Antioxidant, directly detoxifies ROS/reactive nitrogen species (RNS), increases the activity of antioxidative enzymes while suppressing pro-oxidant enzymes in mitochondria, stabilizes the mitochondrial inner membrane
- Xylose - serves as a primer for the formation of heparin/heparin sulfate and chondroitin/dermatan sulfate chains, which is initiated by the attachment of α-D-N-acetylglucosamine (GlcNAc) or β-DN-acetylgalactosamine (GalNAc), respectively. The glucosaminoglycan (heparin/heparan sulfate) and the galactosaminoglycan (chondroitin/dermatan sulfate) chains then assemble by the alternating addition of GlcUA and GlcNAc or GlcUA and GalNAc, respectively.
- Lipoate – Inactivates the nuclear factor kapp B (NF-κB that plays a crucial role in immune response, inflammation, cell growth and survival, and development, acts as powerful antioxidants
- Choline – acts in the synthesis of membrane phospholipids, specifically phosphatidyl choline (PC), which is the predominant phospholipid (>50%) in mammalian membranes. PC is important in maintaining cellular integrity and signaling functions.
- Piperine- the pungent compound contained in black pepper (Piper nigrum L.), Piperine exhibits anti-oxidant, anti-inflammatory and anti-degenerative properties, as well as enhancement of drug absorptionNicotinic acid - water-soluble vitamin and nicotinamide is a derivative of niacin that forms the coenzymes nicotinamide adenine dinucleotide (NAD) and its phosphorylated form, nicotinamide adenine dinucleotide phosphate (NADP). NAD is a key molecule used in the production of energy.
- Isothiocyanates (ITC) - sulfur-containing compounds broadly distributed among cruciferous vegetables, antioxidant, and anti-inflammatory properties.

- myxin - phenazine di-N-oxide which causes enzymatic one-electron reduction, which both donate and accept electrons to other electron transfer molecules for their biological activities.
- Cysteine - an important source of sulfur, which forms the very reactive sulfhydryl (SH or thiol) group for the stabilization of and function of protein and enzymes

The active ingredients of the compounds were combined synthetically resulting in eight (8) new chemical entities (NCEs). Subsequently, each NCE (component) was combined in a 3-NCE component fixed-dose (1:1:1) formulation. The 3-NCE combo (hereafter called **combo**) was designed to address the multifactorial or polypharmacological nature of complex diseases, including restoration and maintenance of a healthy glycocalyx, mitigate excessive oxidative and inflammatory activities and/or target infectious agents *(Fig. 13).*

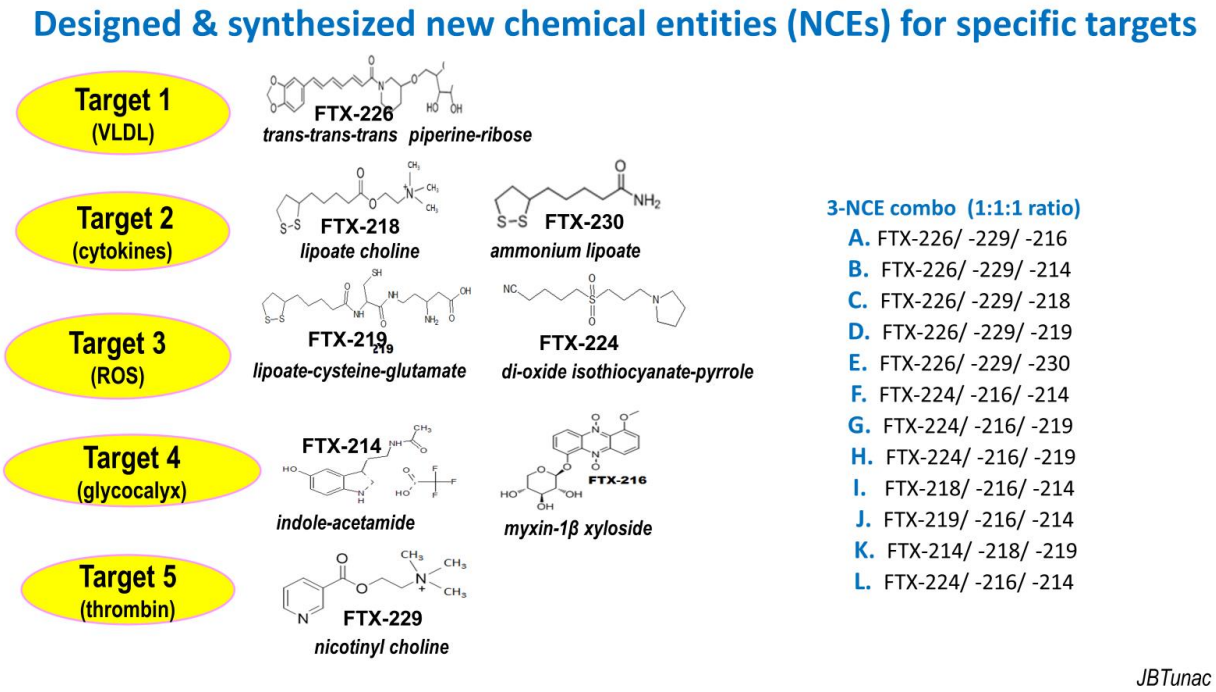

Figure 13. Structures of 8 new chemical entities (NCEs) with corresponding targets and the makeup of the 12 (A-L) different 3-NCE combos (hereafter called combo).

3. Anti-plaque activity of combos using 4 biomarkers as guide for effectiveness.

Each 3-NCE combo was formulated at a ratio of 1:1:1. Anti-plaque activities were evaluated by ELISA using 4 blood markers and subsequently correlated with histopathology. Activities were evaluated in a preventative as well as curative mode: Preventive (PCB added day 7 & 9 to 16-day old mice on fat diet, then drugs added daily, 1 thru 5); Curative (PCB added days 1 & 3 to 16-day mice on fat diet, then drugs added daily, 5 thru 9)

3.1. Hyaluronan Synthase 1 (HAS-1)

The results for Hyaluronan Synthase 1 (HAS-1) are shown in Figure 14. It was observed that the highest HAS-1 levels occurred in the mice on the high fat diet treated with PCB on Day 1 and 3 and sacrificed at Day 4, which was the comparison control for the Curative groups. The comparative control for the Preventative groups were mice on the high fat diet treated with PCB on Day 7 and 9 and sacrificed on Day 11. Reductions in HAS-1 levels were observed in mice treated with:

- Combos G, I and K in the Preventative Protocol, and
- Combos A, B, C, F, I, J, and K (*and L*) in the Curative Protocol.

ANOVA statistics for the comparison of levels revealed no significant changes in the Preventative Protocol, with trends (p=0.18) for both Combos G and K. Statistical differences were observed for Combos A, B, C, F, I, J, and K in the Curative Protocol and ranged from p<0.002 tp p<0.01 (Table 2)

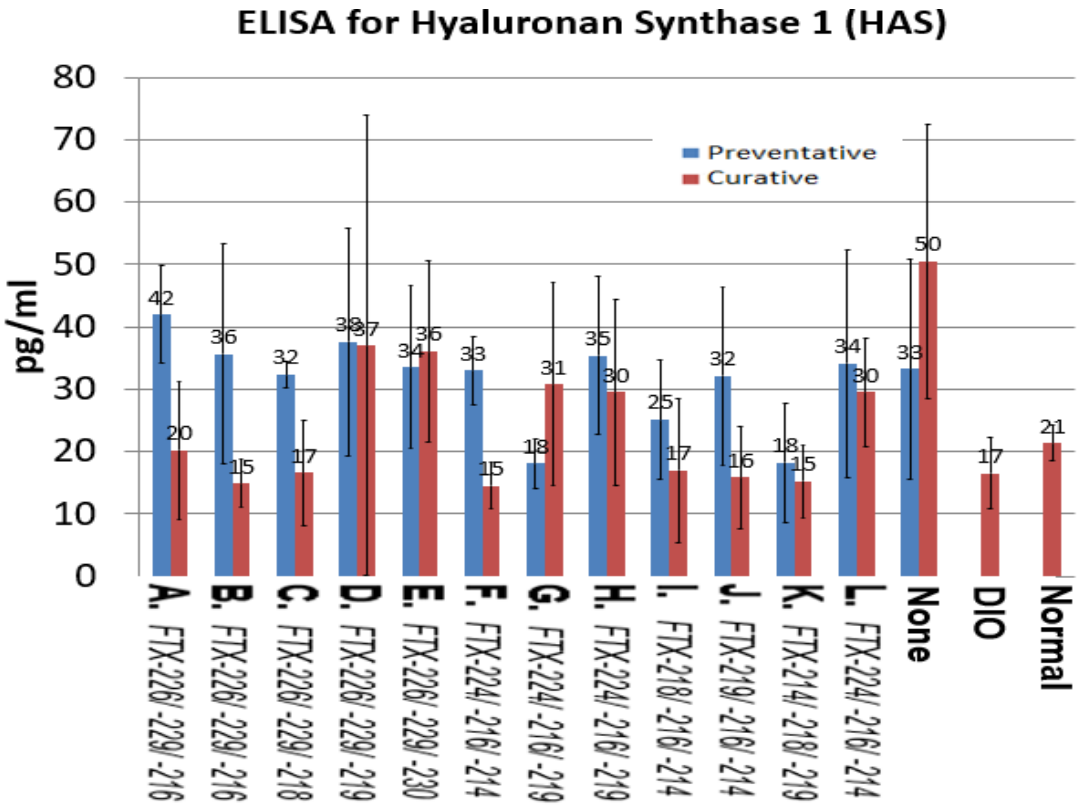

Figure 14. ELISA profile of the 12 combos vs hyaluronan synthase 1 (HAS)

3.2 Heparan Sulfate (HS).

The results for Heparin Sulfate (Hep) are shown in *Figure 15*. It was observed that the highest Hep levels occurred in the mice on the high fat diet treated with Combos A and E in the Preventative Protocol, although high levels were also seen in mice on the high fat diet treated

with PCB on Day 7 and 9 and sacrificed on Day 11, the comparative control for the Preventative groups. Hep levels were not noticeably higher in mice on the high fat diet treated with PCB on Day 1 and 3 and sacrificed at Day 4, which was the comparison control for the Curative groups. Reductions in Hep levels were observed in mice treated with

- Combos F, G, H, I, K and L in the Preventative Protocol, and
- Combos A, B, C, F, J, and K in the Curative Protocol.

ANOVA statistics for the comparison of levels revealed significant changes in the Preventative Protocol, with decreases due to Combos F, G, H, I, K and L ranging from p<0.05 to p<0.001. (Table 2). A statistical difference was observed for Combo C in the Curative Protocol (p<0.05)

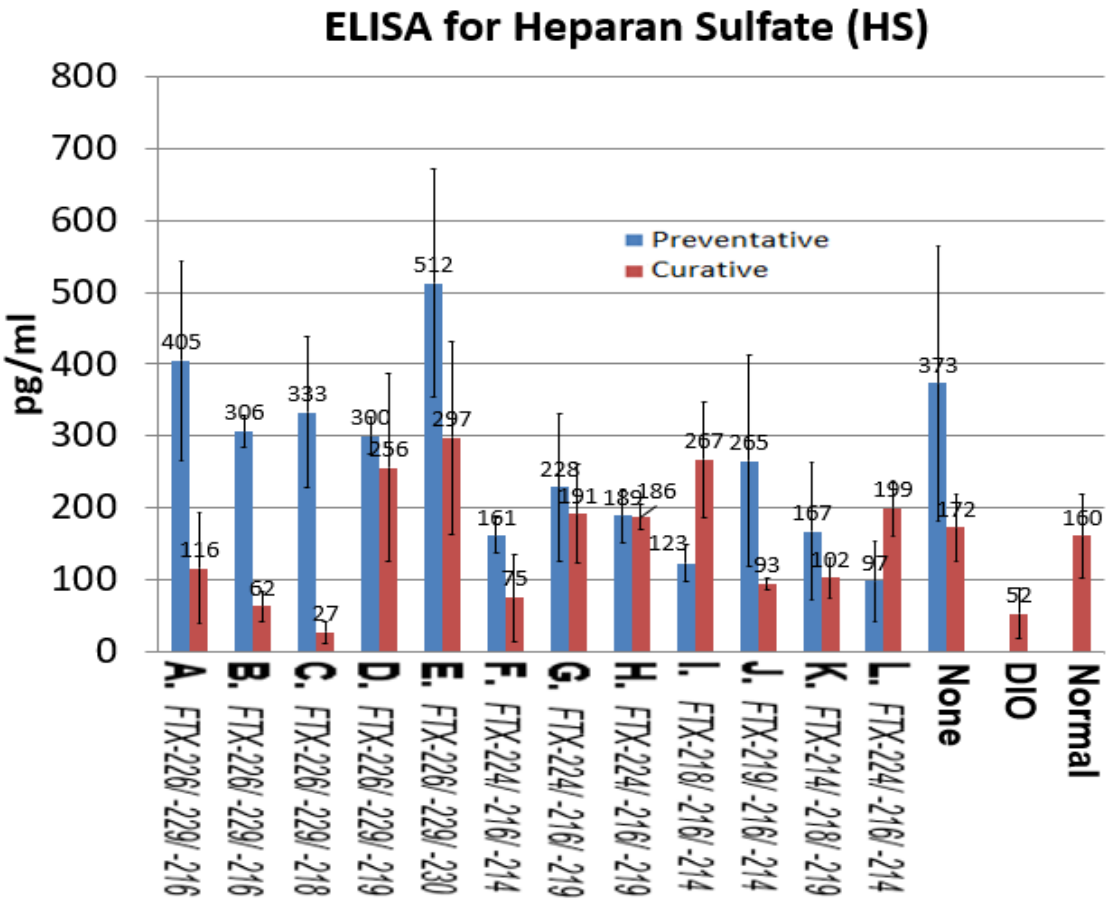

Figure 15. ELISA profile of the 12 combos vs heparan sulfate (HS)

3.3. Total Plasminogen Activation Inhibitor-1 (PAI-1).

The results for Total Plasminogen Activation Inhibitor-1 (PAI-1) are shown in *Figure 16*. It was observed that high PAI-1 levels occurred in the control mice for both the Preventative Protocol and the Curative Protocol. Reductions in PAI-1 levels were observed in mice treated with

- Combos B, D, E, F, H, I, J, K and L in the Preventative Protocol, and all
- Combos except F and J in the Curative Protocol.

ANOVA statistics for the comparison of levels revealed significant changes in the Preventative Protocol, with decrease due to Combos B, D, E, F, H, I, J and L ranging from $p<0.02$ to $p<0.001$ (Table 2). Statistical differences were observed for all Combos except F and J in the Curative Protocol and were typically $p<0.001$ *(Table 2)*

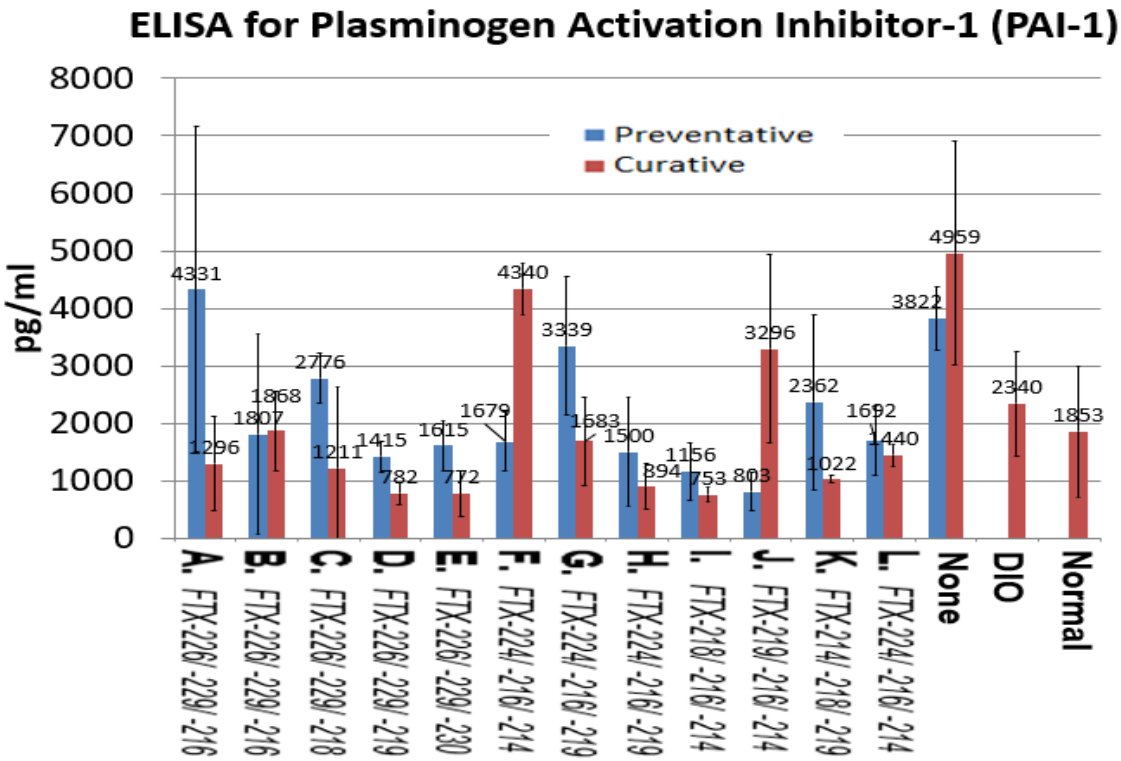

Figure 16. ELISA profile of the 12 combos vs plasminogen activation inhibitor-1 (PAI-1)

3.4. Syndecan 1 (SDC1).

The results for Syndecan 1 (SDC1) are shown in *Figure 17*. It was observed that high SDC1 levels occurred in in mice treated with Combos A, C, E and G in the Preventative Protocol, while the control mice for both the Preventative Protocol and the Curative Protocol did not show notable elevations of SDC1. Nevertheless, Reductions in SDC1 levels were observed in mice treated with

- Combos D, F, J, and K in the Preventative Protocol, and
- Combos B, C, D, E, F, H, I, J, and K in the Curative Protocol.

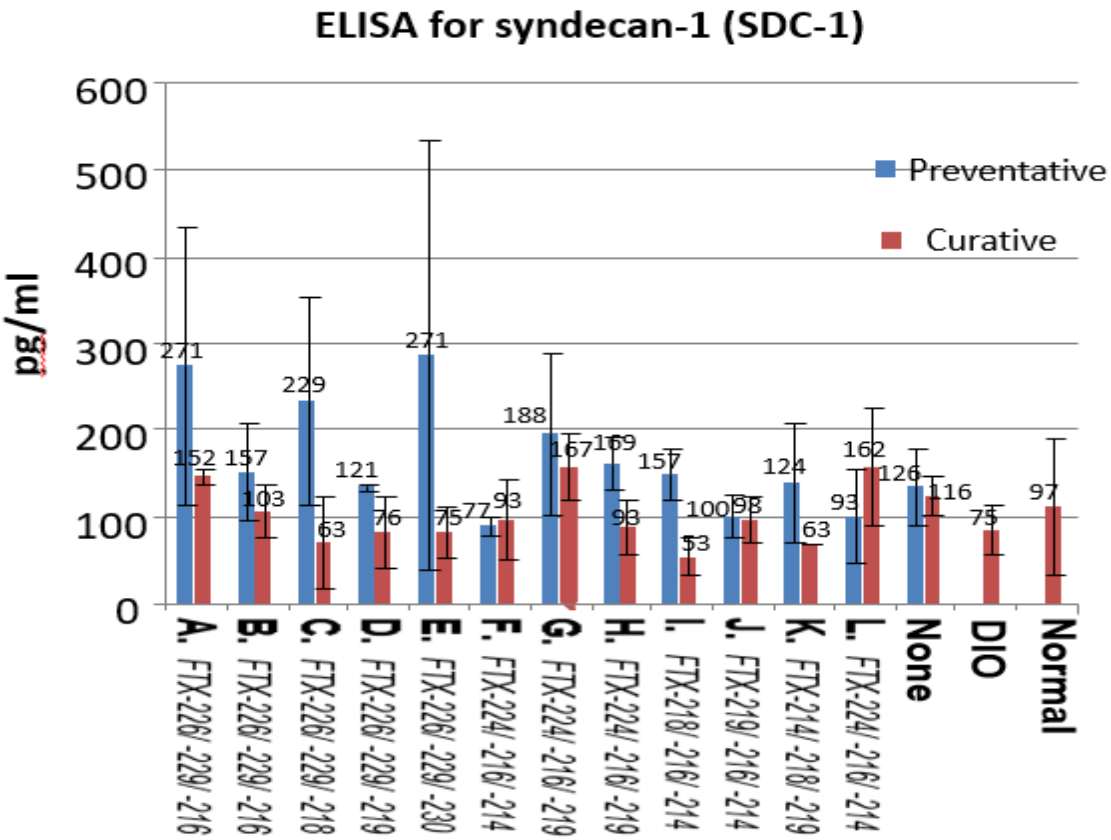

*Figure 17. ELISA profile of the 12 combos vs **syndecan-1 (SDC-1)***

Table 2. Statistics for Biomarker Assays (p values)

Protocol	Drug	Group	Has1	Hep	PAI	SDCI
Preventative	A	1	0.439	.652	.552	0.023
	B	2	0.833	.337	.023	0.771
	C	3	0.931	.556	.224	0.100
	D	4	0.700	.291	.006	0.982
	E	5	0.983	.051	.012	0.015
	F	6	0.979	.003	.015	0.455
	G	7	0.181	.041	.572	0.314
	H	8	0.849	.010	.008	0.655
	I	9	0.470	.001	.003	0.815
	J	10	0.920	.122	.001	0.570
	K	11	0.184	.004	.091	0.942
	L	12	0.938	.001	.015	0.572
Curative	A	14	0.009	.417	.001	0.713
	B	15	0.002	.115	.001	0.772
	C	16	0.004	.037	.001	0.370
	D	17	0.232	.233	.001	0.491
	E	18	0.202	.077	.001	0.490
	F	19	0.002	.138	.469	0.641
	G	20	0.083	.792	.001	0.588
	H	21	0.065	.844	.001	0.542
	I	22	0.004	.186	.001	0.245
	J	23	0.003	.254	.055	0.646
	K	24	0.003	.315	.001	0.345
	L	25	0.066	.730	.001	0.574

3.Toxicology.

No mice died during the study, and no adverse effects were noted due to administration of either the PCB or drug suspensions. No notable gross pathology changes were noted attributable to the drug administration.

4. Histopathology

4.1. ELISA values of blood markers were found to correlate with histopathology, showing preventive and curative effects of the combo NCEs. Since the mouse coronaries were too small, we focused on histopathology in the brachiocephalic artery. Typical histopathology shows plaque formation in control (no NCE treatment) while no plaques in the NCE treatment. For example, no NCE treatment (High fat diet, PCB) revealed presence of a pathology consistent with plaque (A,

10x; and B, 40x) with fibrous material loosely attached to the surface of the arterial wall. In contrast, the NCE treated exhibited the typical features of a normal arterial wall (C), *Fig. 18.*

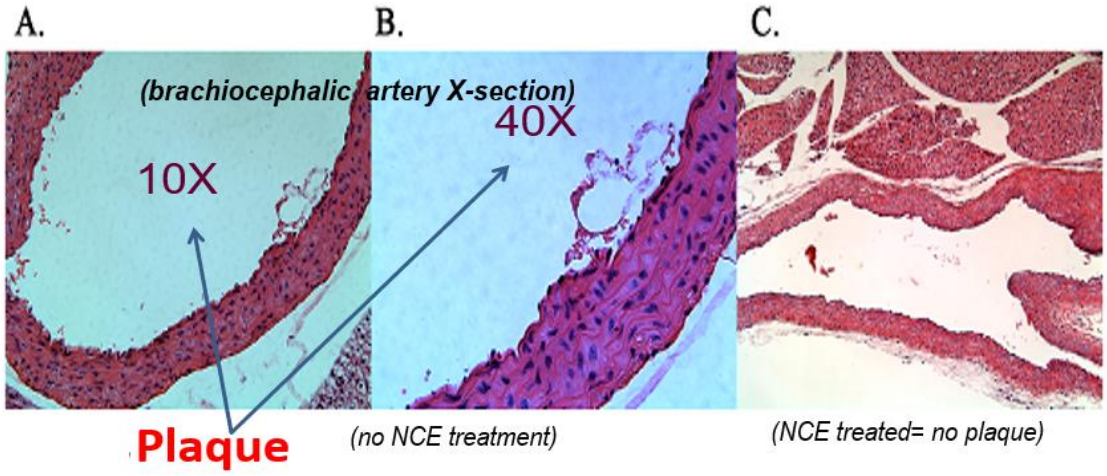

Figure 18. A typical plaque produced in the Tunac arterial plaque (TAP) mouse showing subendothelial site and fibrous cap like that of human.

Other histopathological features in the TAP model (no NCE treatment, high fat diet plus PCB) show various lesion characteristics, including lipid deposits, fatty streaks and eventually a well-defined plaque with fibrous cap *(Fig. 19 c)*.

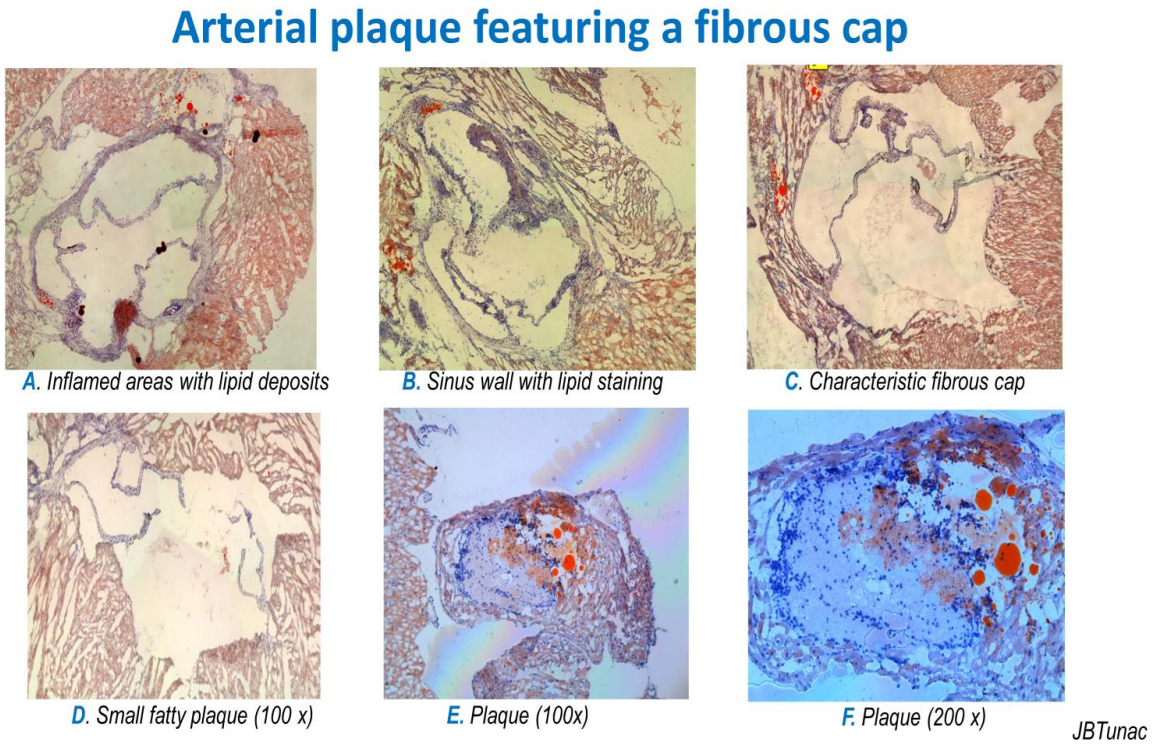

Figure 19. The Tunac arterial plaque (TAP) mouse™I, showing stages of lesion development including lipid deposits, fatty streaks and eventually a well-defined fibrous cap.

Representative histopathologies of other combo treated samples, including C, G, I and L, clearly show normal cell morphologies in both preventive and curative *(Fig. 20)*

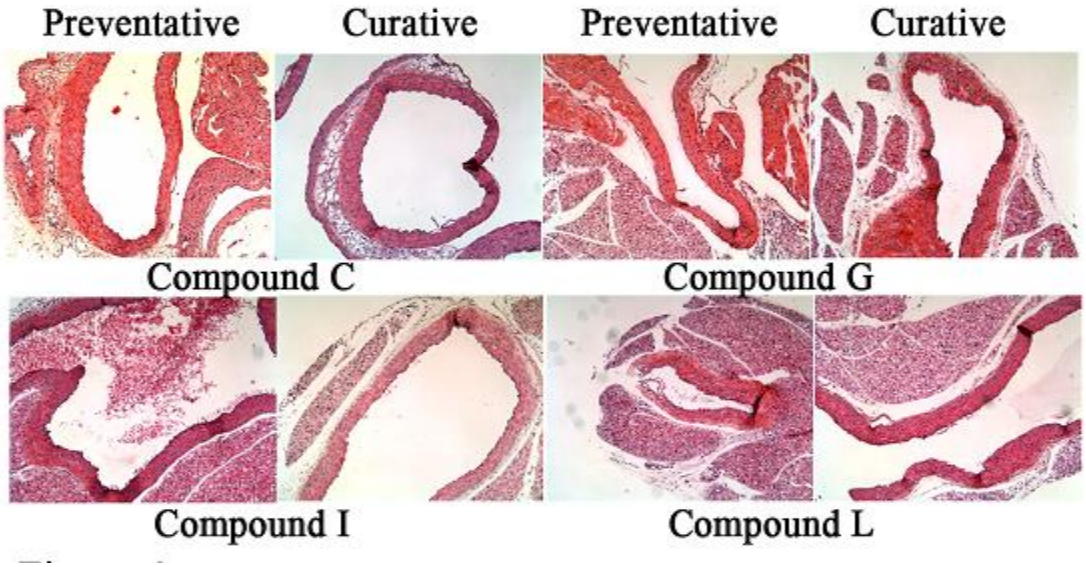

Figure 20. Histopathologies of brachiocephalic arteries from other combo treatments, particularly combo C, G, I and L.

SUMMARY

The 12 combo compounds tested showed varying degrees of activities. While combos A and E were poor performers, combos F, J and K showed excellent activities in reducing glycocalyx disruption as indicated by lower levels of the 4 biomarkers compared to the untreated control. Of the 8 individual components, 5 were recurring components of the active combos, including FTX-214, -216, -218, -219, -224. FTX-214 is common to the 3 combos (combos F, J, K) ; FTX-219 present in 2 (combos K, J) , and FTX-216 and -224 in one (combo F). While combos K and J were active across the board in preventing and curing plaques, combo K for now is chosen as the lead compound and herein named Embotricin™ *(Fig 21).*

3-component compounds curative & preventive of plaque

- *Drugs tested in 3-combo to address multifactor nature of CVD*
- *Drugs active individually, but curative/preventive only in combo!*

3-combo component (FTX)	BLOOD MARKERS (ELISA: pg/ml)								
	Hyaluronan (HAS)		Heparan SO4 (HS)		Plasminogen (PAI-1)		Syndecan-1(SDC-1)		
	preventive	*curative*	*preventive*	*curative*	*preventive*	*curative*	*preventive*	*curative*	
A. 226/229/216	40	20	405	116	4331	1296	271	152	
B. 226/229/214	36	15	305	52	1807	1868	157	103	
C. 226/229/218	32	27	333	27	2776	1211	229	63	
D. 226/229/219	38	37	300	156	1415	782	121	76	
E. 226/229/230	34	36	512	297	1615	772	271	75	
F. 224/216/214	33	15	161	75	1679	4340	77	93	3rd
G. 224/216/219	18	31	228	191	3339	1683	188	167	
H. 224/216/219	35	30	189	186	1500	894	169	93	
I. 218/216/214	25	17	123	267	1156	753	157	53	
J. 219/216/214	32	16	265	93	803	3296	100	98	2nd
K. 214/218/219	18	15	167	102	2362	1022	124	63	1st
L. 224/216/214	34	30	97	199	1692	1440	93	162	
Control *(no drug)*	33	50	373	172	3822	4959	126	116	

- **Combo K** (*Embotricin™), first anti-embolic™ compound:*
  prevents formation of emboli (clots) involving plaque reduction and/or restoration of disrupted endothelial glycocalyx

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Figure 21. Comparative activities of the 12 combos vs the 4 biomarkers with combos J and K showing activities across the board.

Histopathology of K (Embotricin), the lead compound, which is curative, and preventive of plaque shows a normal morphology after drug treatment *(Fig. 22).*

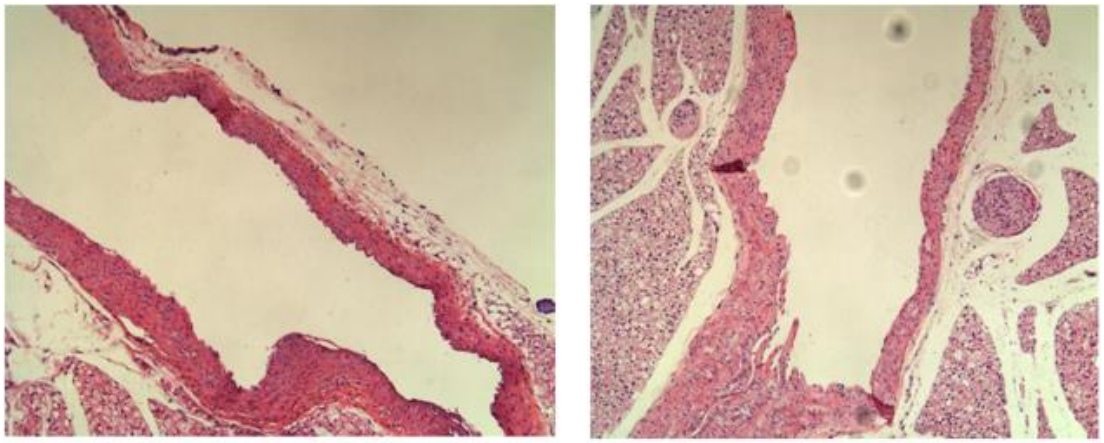

Figure 22. Histopathology of brachiocephalic artery treated with Embotricin showing normal morphology

DISCUSSION

CVD is a multifactorial disease, and we identified the key factors involved in the pathogenesis and rationally synthesized compounds to target each factor. Moreover, in the spirit of polypharmacology, we combined these compounds, specifically a 3-combo component, to capture synergistic effect. Further we evaluated these combos in a natural animal model (TAP mouse), that closely mimics human's daily lifestyle and exposure to pollutants. From such experiment, we identified a series of combo drugs that were preventive and curative of plaques, particularly combo K, with backup combos in J and F. The common components of these combos include FTX-214, -216, -218, -219, -224, with combo K consisting of FTX-214, -218, and -219, herein called Embotricin™. The combo ratio was 1:1:1 and it is possible that optimizing the ratio may well further improve its activity.

Our concept of polypharmacology in CVD starts with the consumption of excess dietary fats, which increase blood viscosity. Thus, a diet of saturated fatty acid was associated with a higher CHD risk (2016. Arterios Thromb Vasc Biol. 36:2011–2018), while a diet of low-fat reduced CVD (2014. J Human Hypertension 28: 170–175), which is consistent with the classic Mediterranean diet (2017.Diabetes Spectrum 30:72-76). Every animal-based food contains both cholesterol and fats; while the cholesterol content is constant (except egg yolk and cheeses), the fat content varies with beef averaging 9.6% fat. The Western type diet averages 21% fat and 0.15% cholesterol *(Fig. 23)*

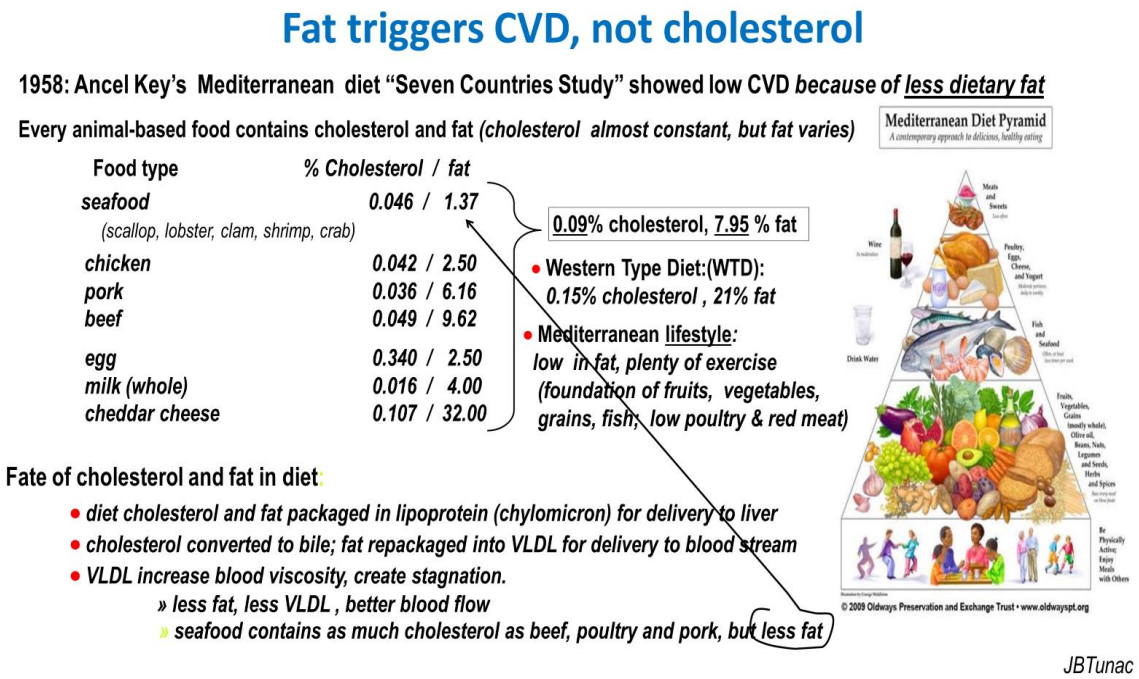

Figure 23. Animal-based diet contains both cholesterol and fat, but fat content varies with cheese and beef as highest. The 21% Western diet associated with heart disease.

Fats or triglycerides and liver-synthesized cholesterol are packaged in VLDL and high, VLDLs constitutes one of the major risk factors for the development of atherosclerosis (2002.Circulation. 106: 2137–2142).

Thus, high-fat meals cause endothelial dysfunction and mild inflammation in the vessel walls (2014. Nutr J 13: 12); even just one fat meal load in healthy young men would show reduced coronary blood flow (2002. Ann Intern Med 136: 523-528). Increased blood viscosity is a major risk factor for cardiovascular event (2000. Eur Heart J 21:515) and VLDL is the most atherogenic triglyceride remnant lipoprotein (TGRL) in the circulation. (2014 Atherosclerosis 236: 244-250].

Thus, high viscosity slows down blood flow, particularly in arterial forks and bends, creating segments of bends or oscillating blood flow and low shear stress (1993. Arterioscler Thromb 13:310). The glycocalyx lining at these sites are typically thinner and prone to injury, which causes endothelial dysfunction and atherosclerosis (2004. Arterioscler Thromb Vasc Biol 24: 12–22). Such bends are found in various parts of the vasculature including the aortic arch, carotid sinus, brain, heart, and limbs (2011. Physiol Rev. 91:327–387), *Fig. 24*

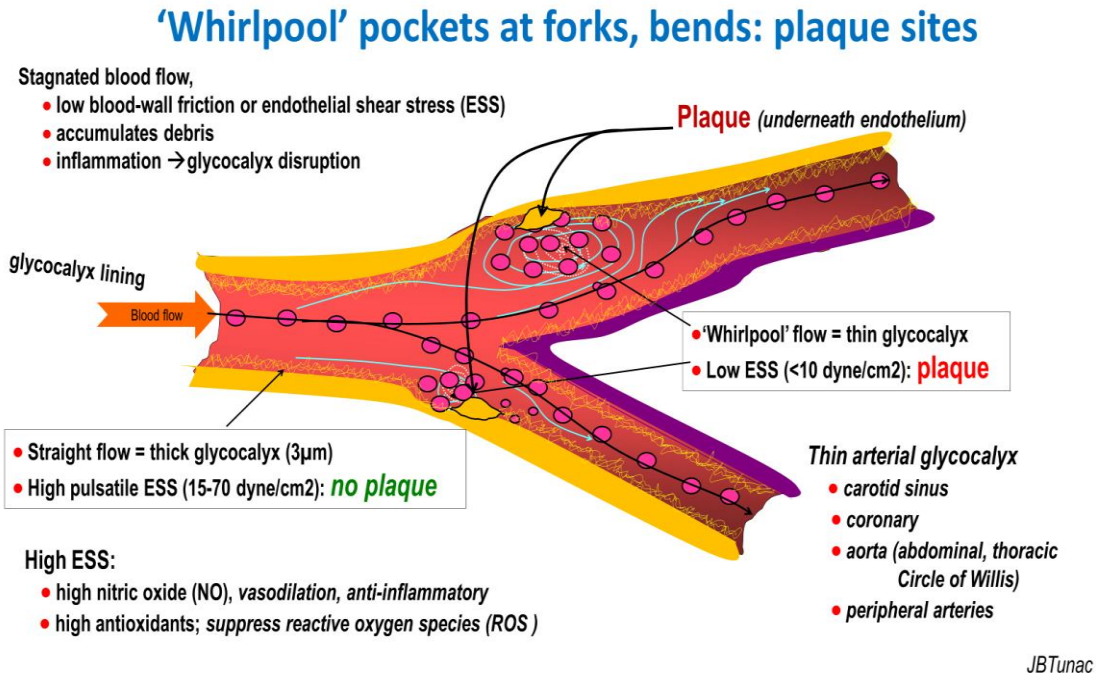

Figure 24, Blood flow slows down at arterial forks and bends, creating a "whirlpool pocket" and plaque site.

Stagnant flow traps chemical pollutants, including particulate matters (PM 2.5) causing oxidation, disruption of protective arterial endothelia glycocalyx (AEG) Proof in point: The collapse of the World Trade Center (WTC) towers on September 11, 2001 (9/11) produced more than a million tons of ~ 1.5% PM 2.5 dusts including gaseous and chemical inhalations. Firefighters arrived on the morning of 9/11 had 44% greater incidence of CVD compared with those who arrived later.and persisted for years after 9/11 (2019. JAMA Net Open 2: e199775).

Disrupted AEG is a first step in the atherosclerosis process, (2016.therosclerosis. 252:136–146), Endothelium disruption allows blood debris and lipoproteins to infiltrate the subendothelial space (1995.Arterioscler Thromb Vasc Biol. 15(5):551–561), which subsequently become oxidized and trigger inflammation. Inflammation involves the recruitment of monocytes across the endothelial monolayer into the intima where they proliferate and differentiate into macrophages (2000. Nature. 407(6801):233–241). The macrophages ingest oxidized debris and lipoproteins, developing into foam cells then mature into plaque (1993. Nature. 362(6423):801–809),

Acute inflammation due to infection or injury predisposes the plaque to rupture. Ruptured plaque triggers clot (embolus) formation, causing stroke (clogged artery to the brain), heart attack (clogged artery to the heart), or PAD (clogged artery to the arms or legs). Arterial bends and plaques are found throughout the vasculature with CHD representing the most death in CVD due to thromboembolism *(Fig 25).*

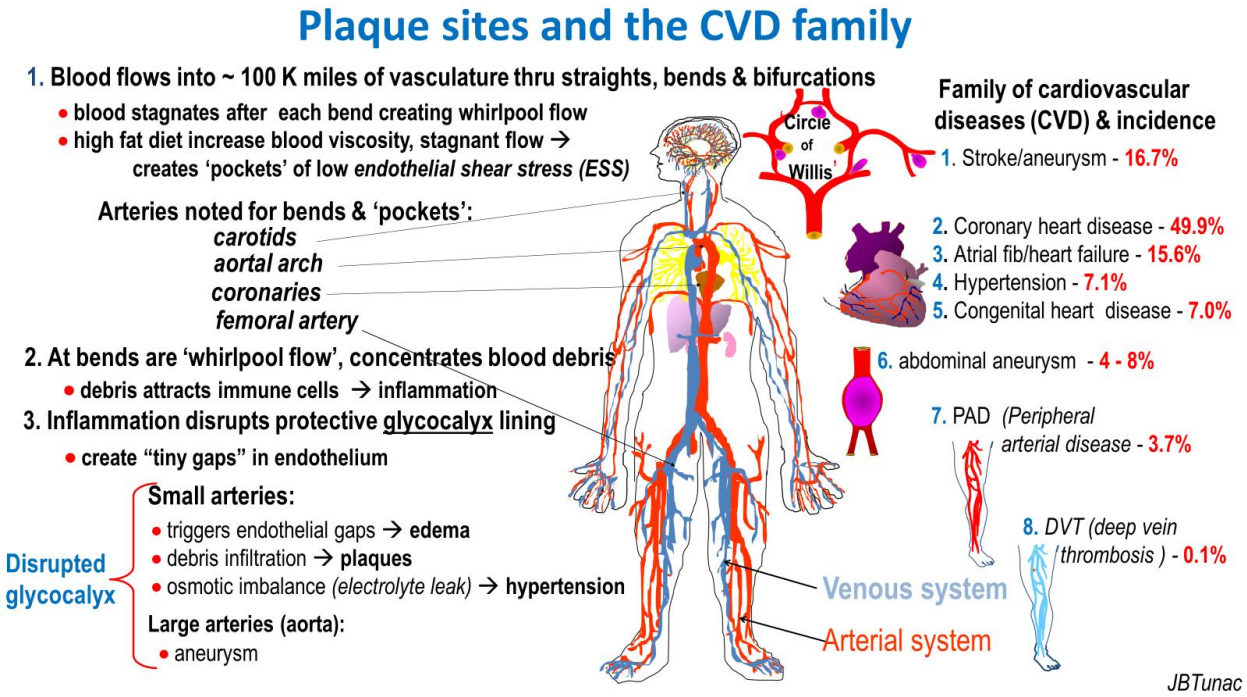

Figure 25. Family of CVD as manifested in different parts of the vascular system.

Thromboembolism is the breakage of amplified (large) clot to become an embolus and clog downstream vessel that is too small to let it pass causing stroke (clogged artery to the brain), heart attack (clogged artery to the heart), or PAD (clogged artery to the arms or legs), *Fig 26.*

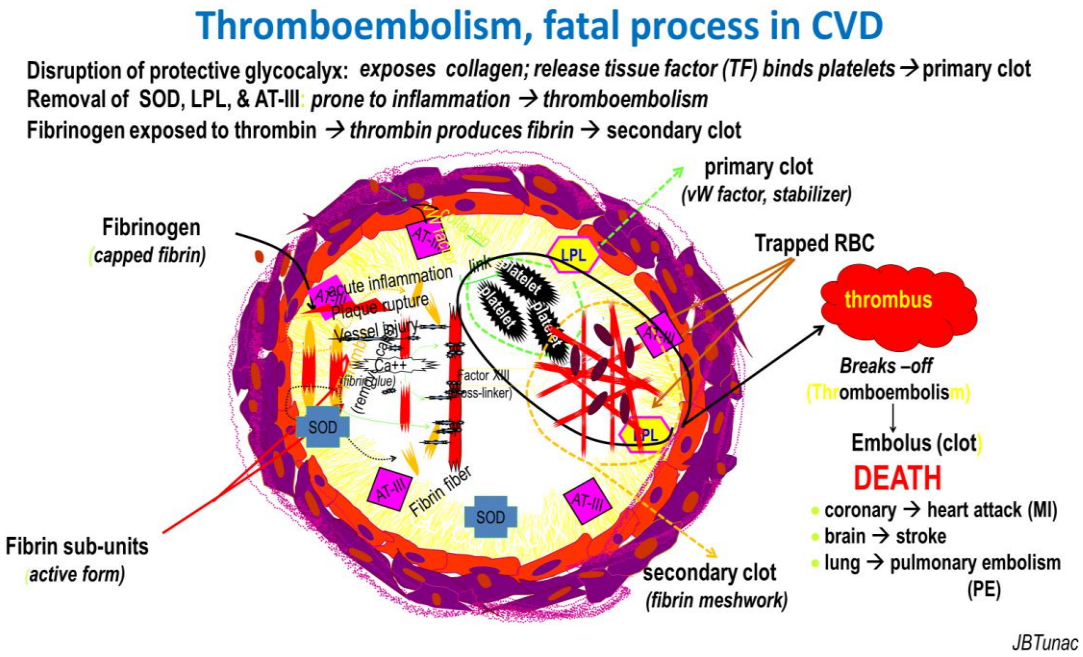

Figure 26. Formation of clot starts with a disrupted glycocalyx (primary clot) and progresses into a secondary clot (embolus), this is the fatal component of CVD

The integrity of the glycocalyx is maintained by a balanced synthesis and shedding of its component parts. While excess shedding is associated with cardiovascular risks, it is possible to reverse shedding; indeed, a number of compounds have been shown to restore glycocalyx and reverse plaque *(Fig. 27).*

Agents with some protective glycocalyx activity

Treatment	Reference
• Hydrocortisone	2007. Anesthesiology. 107:776–84..
• Antithrombin	2009. Cardiovasc Res. 83:388–96.
• Protein C	2008. Shock. 29:572–6
• Nitric oxide	2008. Crit Care. 12(3):R73.
• Hyaluronic acid & chondroitin sulphate	1999.Am J Physiol. 277:H508–14.
• Sulodexide	2010. Diabetologia. 53(12):2646–55.
• Lidoflazine	1983. J Thorac Cardiovasc Surg. 85:758–68.
• Albumin	2009. Transplantation. 87:956–65.
• Hydroxethyl starch	2006. Anesthesiology. 104:1223–31.
• N-acetylcysteine	2006. Diabetes. 55(2):480–6.
• Metformin	2013. Cardiovasc Diabetol. 12:175.

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Figure 27. Published literature describing clinical potential of various compounds in ttreating glycocalyx

Embotricin™ is the first drug that systematically addresses glycocalyx restoration or repair and the other predisposing risk factors of CVD including oxidation and inflammation. The robust preclinical data may well prove Embotricin™ to be the first curative drug against CVD with the same impact as penicillin to infectious disease. While penicillin is the first antibiotic, Embotricin™ is the first anti-embolic™ compound (defined as an agent that prevents formation of emboli (clots) involving plaque reduction and/or restoration of disrupted endothelial glycocalyx). Thus, the proposed mode of action of Embotricin™ fits in the paradigm of polypharmacology *(Fig. 28)*

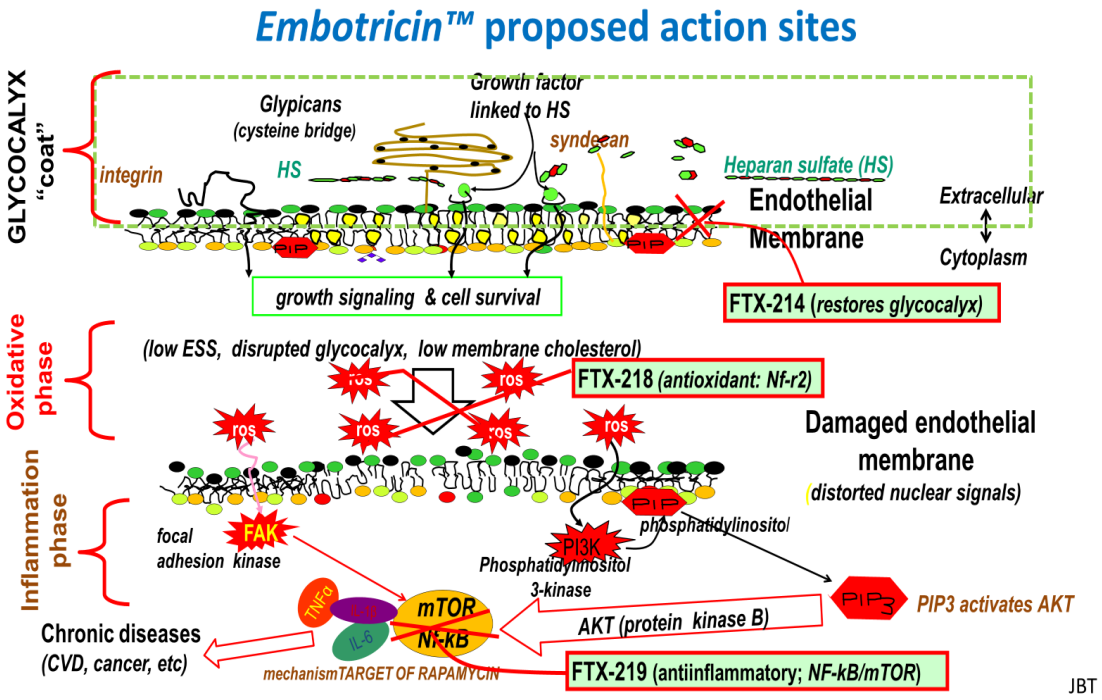

Figure 28 Proposed molecular mode of action of the three NCE components of Embotricinn™

ACKNOWLEDGEMENT
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