Curative and preventive treatment for cardiovascular disease (CVD) targeting multiple etiology

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Abstract

Objective: Gene deficient or knockout (KO) mice and rabbits are models of atherosclerosis focusing on cholesterol plaques, which do not reflect the complex etiology of cardiovascular disease (CVD). One-drug-one-target, paradigm has produced the statins but are at best palliative. CVD is of a multiple etiology and this study aims to develop a combo-compound therapy complementing the multifactorial nature of the disease.

Methods and results: A conceptual thromboembolic pathway were constructed to reflect the multiple etiology of CVD. Druggable sites were identified and new chemical entities (NCEs) synthesized to match target sites, then a series of 3-NCE combos were designed to address in toto the thromboembolic pathway. A natural mouse was created to produce plaques and plaque reduction was the end point to evaluate the curative and/or preventive treatment effect of the 3-NCE combos. Histopathology monitored the presence of plaque, but a 4-panel biomarker, based on glycocalyx disruption, was subsequently developed as a surrogate to monitor plaque formation. Of the 12 different 3-NCE combos, four were found to be both curative and preventive. One combo was chosen and given the designation Embotricin™.

Conclusions: CVD and its multiple etiology is preventable and curable adapting a combo-drug therapeutic platform.

Keywords: *Embotricin™, CVD, combo therapy, KO mice, glycocalyx, pharmacognosy, polypharmacology, epigenetics, lipoprotein, thromboembolic, xenoplexic disease, chronic disease, ROS, NCE, TAP, Heart failure, Particulate matters, arterial plaque, biomarkers, diagnostic, FDC (fixed dosed combo); GCX (glycocalyx); NCE (new chemical entity); GAG (glycosaminoglycan); AEG (arterial endothelial glycocalyx); RBC (red blood cells); HTN (hypertension); ETS (electron transport system); ROS (reactive oxygen species); xenobiotics; apoptosis; necrosis; pyroptosis; autophagy; TAG (triglycerides); VLDL (very low density lipoprotein); LDL (low density lipoprotein); LDL-R (LDL receptor); HDL (high density lipoprotein); FH (familial hypercholesterolemia); CETP (cholesteryl ester transport protein); TGRL (triglyceride remnant lipoprotein); ESS (endothelial shear stress); PM (particulate matter); LAD (left anterior descending); CHD (coronary heart disease); thromboembolism; thrombosis; embolism; ATE (arterial thromboembolism); VTE (venous thromboembolism); PAD (peripheral arterial disease); NIH (national institutes of health); AHA (American heart association); NCEP (national Cholesterol Educational Program); KO (knock out); GPCR (G-protein coupled receptor); HDAC (histone deacetylase); ApoE; PCSKG; POPS (persistent organic pollutant); PCB (polychlorobiphenyl); ELISA (Enzyme-linked Immunosorbent Assay)*

HIGHLIGHTS

- Vascular diseases including CVD are triggered by extraneous (xeno) factors, which is of multiple (plexic) origin including environment, lifestyle, and drug usage, herein called xenoplexic diseases. Xenoplexic disease is an etiologic description while chronic disease is symptom-centric.
- Constructed a xenoplexic overview of CVD, identified 3 druggable targets and synthesized new chemical entities (NCEs) to address these targets.

- Designed a mouse model to reflect the natural progression of CVD in humans and evaluated the NCEs as a 3-combo therapy. Histopathology showed subendothelial location of plaques with well-defined fibrous caps.
- Developed a 4-panel “diagnostic fingerprint”, which served as a histopathology surrogate in monitoring plaque formation. The biomarkers are remnants or detritus from glycocalyx disruption including, heparan sulfate (HS), hyaluronan synthase (HAS), syndecan-1 (SDC-1), and plasminogen activator inhibitor-1 (PAI-1).
- Tested several 3-NCE combos vs the mouse model and identified 4 promising combo leads that prevented and reversed plaques, namely Combos K, J, F and D. Combo K (3-NCE combo of FTX-214, -218, -219) is chosen as the lead candidate and hereby designated as *Embotricin*™

1. INTRODUCTION

1.1. Incidence of cardiovascular disease (CVD). Cardiovascular disease (CVD) refers to a group of disorders that includes hypertension, coronary artery disease, peripheral artery disease, stroke, congenital heart disease, and heart failure **(1)**. 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 for a third of all deaths and half of all non-communicable-disease-related deaths. The available drugs vs CVD are palliative at best because they are symptom targeted. There is no curative drug against this disease because the pervading one-drug-one disease paradigm is symptom-centric, which develop one drug to target a symptom. For example, diuretics, angiotensin converting enzyme (ACE) inhibitors, angiotensin receptor blockers (ARBs), calcium antagonists, and beta blockers to treat symptoms of hypertension; statins, bile-sequestrants, fibrates, niacin to treat lipidemia/hypercholesterolemia; and antiplatelets, anticoagulants, fibrinolytics for blood pooling.

1.2. Trends in drug development

1.2.1. Pharmacognosy and early medicines. Pharmacognosy is the exploitation of secondary metabolites found in natural products, including plants, animals, and microorganisms as sources of pharmaceuticals **(2).** The effectiveness of plant extracts (folk medicine) as early medicines were due the synergistic effect of complex mixtures of molecules.

However, in the second half of the 20th century, progress in science allowed the purification and isolation of active ingredients. For example, the isolation of morphine (1804), quinine and colchicine (1820), atropine (1833), and cocaine (1860), shifted drug research into the “one drug-one target-disease" philosophy **(3)** assuming that these were the ‘one-component’ responsible for activities. This paradigm prompted the mass screening of microbial metabolites in the ‘40s searching and isolating that ‘one-component’, which paid dividends marking the golden era of antibiotics **(4).** Microbial metabolites were subsequently exploited for chemotherapeutic agents **(5),** which yielded number of anticancer agents **(6).**

1.2.2. Genomic drug target. The ‘one drug-one target’ complements the ‘one gene-one enzyme’ concept of Tatum and Beadle in the early 1940s **(7).** The premise is to identify a specific enzyme associated in the disease ontology and inhibits its activity. For example, screening of 3-hydroxy-3-methylglutaryl coenzyme

A (HMG-CoA) reductase (key enzyme in cholesterol biosynthesis) yielded the parental statin compactin **(8).** Subsequently, the Human Genome project promised some 30,000 genes as "druggable" targets **(9)** However, finding the target gene for a 'magic-bullet' has proven to be unsuccessful **(10),** because > 90% of disease risks are due to lifestyle and environments **(11).** For example, genetic factors only make a minor contribution to chronic diseases **(12)** while the majority accounted for by environment **(13).**

*1.2.3. Epigenetic and chronic diseases***.** Environmental exposures and lifestyle modulate gene expression through epigenetic processes **(14).** Genes, studied under genetics, are basic units of heredity described by a specific nucleotide pattern; the changes in gene activity during development are studied under epigenetics **(15).** Epigenetic changes can be a cellular (stem cell) or a molecular (cell division) phenomenon **(16).** The predominant epigenetic mark is methylation at cytosine in 5'-cytosine-phosphodiester bond-guanine-3' (CpG) dinucleotides to produce 5-methylcytosine (5mC) **(17)** during oxidative stress **(18),** which is temporally stable for weeks and elicit distinct biological responses **(19)** or passed through cell division with the help of DNA methyltransferases (DNMTs) as heritable epigenetic change. In CVD, epigenetic changes start with endothelial disruption **(20).**

*1.2.4. Polypharmacology and diseases***.** The 'one drug-one target' platform assumes that a drug with a specific target does not have off-target side-effects. However, a single drug could be promiscuous, interacting with 6-28 targets **(21).** In this regard, polypharmacology was conceived almost 25 years ago to exploit the promiscuity of a single drug **(22)** acting on multiple targets of a disease pathway, or single drug acting on multiple targets pertaining to multiple disease pathways **(23)**. Sunitinib exemplifies polypharmacological drug targeting, which activates the multiple receptor tyrosine kinases (RTKs) becoming the first drug to be approved for two separate indications simultaneously, including gastrointestinal and kidney cancers **(24).**

An extension of polypharmacology is 'systems pharmacology' (or 'systems therapeutics'), aimed at targeting biological networks rather than single transduction pathways **(25)** and the products are referred to as multi-target or systems pharmacology drugs **(26).**

*1.2.5. Precision medicine***.** An evolution of polypharmacology is precision medicine **(27).** The term 'precision medicine' was first coined in 2009 **(28),** which tailors medical treatment with medical decisions, treatments, practices, or products to a subgroup of patients, instead of a one-drug-fits-all model **(29).** Thus, diagnostic testing is employed to customize appropriate and optimal therapies based on a patient's genetic content or other molecular or cellular analysis **(30),** which includes molecular, imaging, and analytics **(31).**

*1.2.6. Combination drug treatment***.** In the golden era of antibiotics, these drugs were initially prescribed as monotherapies and then expanded to fixed-dose combinations (FDC). FDC was championed by the head of the FDA's Division of Antibiotics, Henry Welch, who endorsed Sigmamycin (tetracycline and oleandomycin: Pfizer) and Panalba (tetracycline/novobiocin: Upjohn) **(32).** While Sigmamycin components demonstrated clear synergic action **(33),** Panalba's two active agents mutually inhibited each other and decreased efficacy **(34),** which prompted an FDA review and eventual recall of this FDC in late 1959.

The efficacy requirement for FDA drug approval was eventually embedded in the Kefauver–Harris Amendments of 1962 before approval of FDCs **(35).** Compliant with the efficacy amendment, Bactrim (sulfamethoxazole and trimethoprim) was approved by the FDA in 1973 as the first antibiotic FDC and has become a drug mainstay in the treatment of ear infections, bronchitis, pneumonia, urinary tract infections (UTIs), intestinal infections, traveler's diarrhea **(36).** Bactrim components work synergistically through consecutive steps in bacterial folate metabolism halting the growth of bacteria **(37).**

Effect of combined drugs could be antagonistic, additive, or synergistic **(38).** Currently, the increased number of monotherapy drugs that lack efficacy is the driver for combination drugs to search for synergy. Synergistic drug combinations have numerous advantages over monotherapy, including increased efficacy, decreased dosage with equal efficacy, reduced side effects and reduced drug resistance **(39).**

1.3. The molecular roots of CVD

1.3.1. Dysfunctional blood flow: The cardiovascular system starts with the heart, which pumps oxygenated blood through branching arteries, arterioles, and capillaries to tissues. Deoxygenated 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 is a negatively charged, ~0.5–5 μm, layer lining the microvasculature, which allows negatively charged red blood cells (RBC) to glide through the narrow capillary beds **(40)**. GCX disruption due to environmental factors result in dysfunctional blood flow and a sequela of CVD **(41).**

The outer ornamentation of GCX are sulfated glycosaminoglycosides (GAG), notably heparan sulfate (HS). HS confers negative charge for GCX as well as the red blood cells (RBC) and negative charges repel, which allows a frictionless flow of RBC through the GCX lining **(42).** However, when the GCX surface of either units is breached, Na^+ ions are exposed (normally buried in the GCX bed), creating 'positive patches' and disrupts blood flow causing hypertension (HTN) **(43).** Moreover, disruption of the GCX exposes other electrolytes embedded on either side of the membrane triggering a family of CVD diseases **(44).**

1.3.2. Disruption of Glycocalyx (GCX) affects blood flow. All cells in the human body are covered by GCX, including the endothelium of arteries called the arterial endothelial glycocalyx (AEG). AEG is multifunctional, serving as a protective lining, a nest to blood-flow regulating components **(45),** filters off cell debris, acts as a sensor to changes in the microenvironment and then regulates vascular tone, level of circulating cell adhesion molecules, coagulation, fibrinolysis, and inflammation of the vessel wall **(46).** Disruption or dysfunction of AEG is a first step in the atherosclerosis process, which is reflected by shedding of components (detritus) including proteoglycans and glycoproteins **(47).** The glycoproteins are protein-glycan conjugates that make up three families of adhesive molecules, which include the selectin family, the integrin family, and immunoglobulin superfamily that act as a mechanotransductor of shear stress **(48).** Damage to the glycocalyx precedes vascular pathology **(49).**

1.3.3. Food and environmental pollutants produce oxidants. Food is metabolized to extract electrons from hydrogen and shuttled through the electron transport system (ETS) by the electronegative oxygen to

generate energy. However, ~2% electrons leak from the ETS and reacts with molecular oxygen **(50).** Sequential reduction of the two unpaired electrons of molecular oxygen leads to formation of a family of reactive oxygen species (ROS), which in excess are oxidative. Optimum ROS levels are beneficial, which is maintained by built-in antioxidant enzymes **(51).** However, overeating **(52),** high fat diet **(53)** and exposure to stressful environment produce extraneous ROS, which favors an oxidative balance **(54, 55, 56).** Chronic exposure to environmental chemicals, including persistent organic pollutants, volatile and semi-volatile organic compounds, radiation, pathogens, allergens, and psychological stress, generates extraneous ROS leading to GCX disruption **(57)** ***(Fig. 1)***

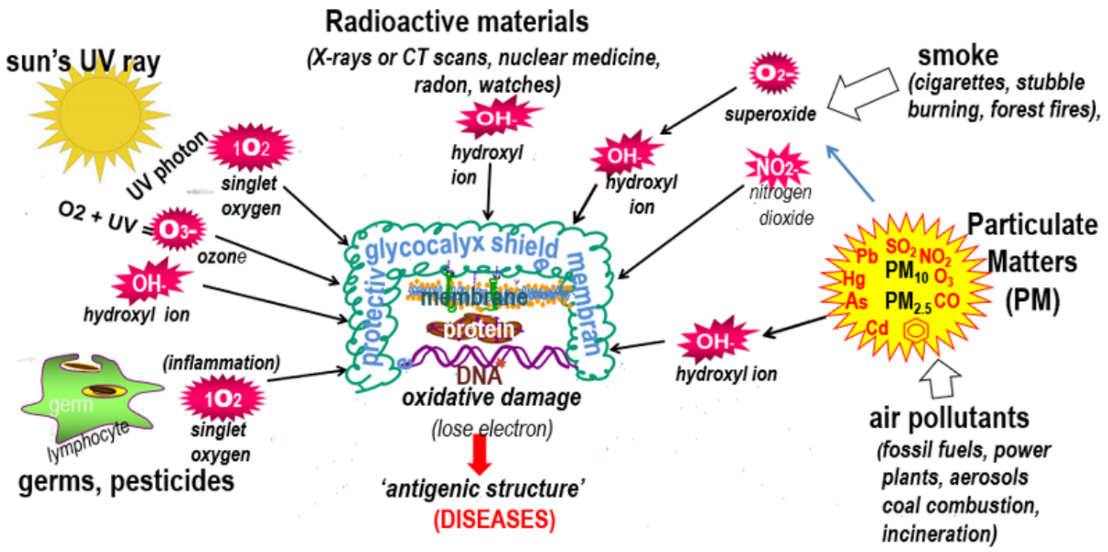

Figure 1. Environmental factors generate reactive oxygen species (ROS) and disrupt the cells protective coat glycocalyx.

Oxidative damage to GCX **(58)** generate debris or detritus, which become antigenic resulting in inflammation and additional ROS. The antigenic molecules triggers inflammation by the release of histamine, an organic nitrogenous compound stored in mast cells and basophils, which binds to various cellular receptors. Histamine increases the permeability of the capillaries to white blood cells and some proteins, to engage with the antigens (pathogens, xenobiotics) **(59).** In distressed tissues, histamine regulates the fate of the cell **(60)** and failure to recover from distress result is cell death (i.e., apoptosis, necrosis, pyroptosis, or autophagic cell death) **(61).** In this regard, modulation of histamine is a therapeutic strategy for the prevention or treatment of CVD **(62).**

1.3.4. High fat diet creates viscous blood. Fats or triglycerides (TAG) are the best source of energy (9 calories/gram) but not soluble in water, hence they are packaged in lipoprotein for delivery to tissues. Very low-density lipoprotein (VLDL) packages TAG along with liver-produced cholesterol and once the fat cargo is unloaded, VLDL becomes low-density lipoprotein (LDL), which is the desired lipoprotein that delivers cholesterol to tissues via the LDL-receptor (LDL-R) **(63).** If the LDL-R is compromised, either by AEG disruption or genetic, LDL cannot be delivered to tissues and consequently a buildup of LDL. For example, Brown and Goldstein discovered a genetically defective LDL-R causing familial hypercholesterolemia (FH) **(64).** Because of this discovery, LDL, has since been labeled 'bad cholesterol'. Essentially all LDL particles

are derived from VLDL and all VLDL goes to LDL **(65).** On the other hand, high density lipoprotein (HDL) is mainly secreted by the liver (70-80%) and small intestines. HDL exchanges TAG via cholesteryl ester transfer protein (CETP) or plasma lipid transfer protein (PLTP) with VLDL and LDL **(66)**, giving HDL the moniker as "good cholesterol".

A high TAG diet means high VLDL, resulting in viscous blood and risk of CVD death **(67)**. VLDL is the most atherogenic triglyceride remnant lipoprotein (TGRL) **(68)**, which rapidly penetrates the arterial wall, increases endothelial inflammation, and facilitates the infiltration of monocytes forming foam cells resulting in atherosclerosis **(69).** A diet of saturated fatty acid is associated with a high CHD risk **(70)**; even just one fat meal load in healthy young men reduces coronary blood flow **(71).**

On the other hand, low TAG diet reduces incidence of CVD **(72)**, which is consistent with the Mediterranean diet **(73)**. Every animal-based food contains both cholesterol and fats with a constant cholesterol content (except egg yolk and cheeses) while the fat content varies, with beef averaging 9.6% fat **(74)**; the Western diet averages 21% fat and 0.15% cholesterol. Cholesterol in the diet does not cause atherosclerosis because liver-produced cholesterol 7α-hydroxylase (CYP7A1) converts it into bile **(75).** Most of the cholesterol supply in the body are synthesized de novo, which is tightly regulated to prevent over-accumulation and abnormal deposition within the body **(76).** For these reasons, cholesterol intake in food has little, if any, effect on total body cholesterol content or concentrations of cholesterol in the blood **(77).**

1.3.5. Viscous blood creates whirlpool pockets and low shear stress. The straight arterial segment allows unimpeded blood flow typical of high endothelial shear stress (ESS) and a thick protective GCX layer. On the other hand, segments of bends or bifurcations with oscillating blood flow have low ESS **(78),** typically thinner GCX lining and prone to endothelial injury and atherosclerosis **(79).** Such bends are found in the aortic arch, carotid sinus, brain, heart, and limbs **(80).** On the other hand, the descending thoracic aorta, where blood flow is uniform and unidirectional with high ESS, does not form plaque **(81).** Normal and high ESS are atheroprotective being involved in compensatory remodeling, but when remodeling fails, the plaque intrudes into the lumen **(82).**

Low ESS are generated in whirlpool pockets, which traps environmental pollutants and debris including dead cells, 'microbial contaminants' and particulate matters (PMs) e.g., chemicals, smoke, pollutants, particulate matters. PMs, particularly $PM_{2.5}$ trigger oxidative stress, inflammation and endothelial dysfunction **(83).** These, factors combine to further compromise a fragile GCX at such sites, exposing the endothelium to injury creating 'tiny gaps', subsequently blood debris and macrophage infiltration to form plaque ***(Fig. 2)***.

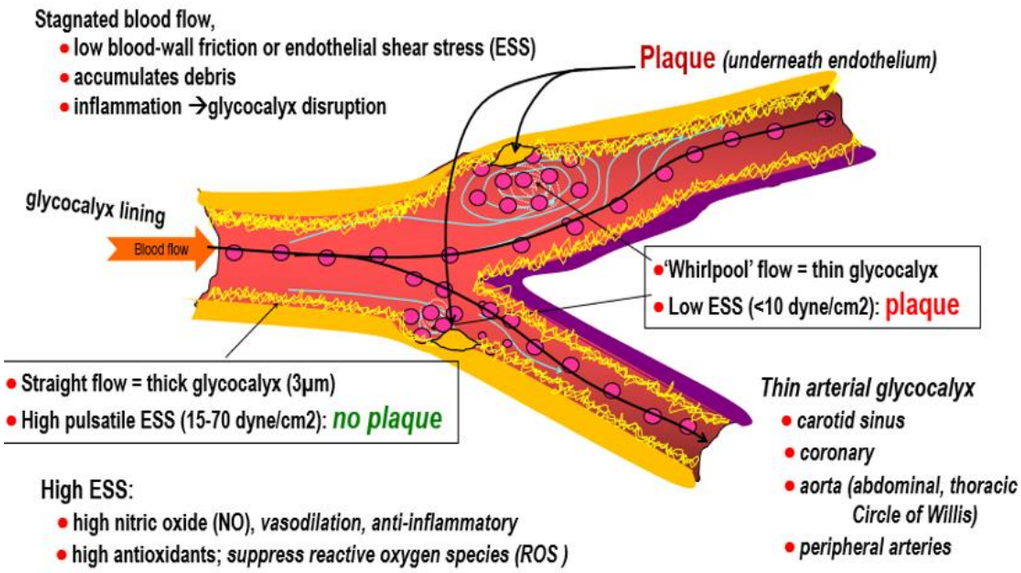

Figure 2. Blood flow slows down at arterial forks and bends, creating a "whirlpool pocket" and plaque sites

1.3.6. Coronary arteries have most bends and plaques. The carotid sinus and coronary arteries are plaque prone because they have the most bends and bifurcations. Of the coronary arteries, the left anterior descending (LAD) has at least 4 bends and as many plaques accounting for coronary heart disease (CHD) as the most prominent of CVD cases **(84).** Acute infection or injury **(85).** ruptures vulnerable plaques, which triggers thromboembolism **(86).** Thrombosis and embolism are similar but occur in unique conditions: thrombosis occurs when a thrombus, or blood clot, develops in a blood vessel and reduces the flow, while embolism occurs when a piece of a blood clot, foreign object, or other bodily substance becomes stuck in a blood vessel and obstructs blood flow. Thromboembolism is the obstruction of vessel beds by embolic material derived from a thrombus from a distant site which could be arterial thromboembolism (ATE) or venous thromboembolism (VTE) that includes deep-vein thrombosis (DVT) **(87).** Thrombus formation on a fissured or ruptured plaque is the main pathogenetic mechanism for unstable angina and the acute coronary syndromes including stroke, heart attack and peripheral arterial disease (PAD) **(88, 89, 90, 91, 92)** with CHD accounting for 47.7% death ***(Fig. 3).***

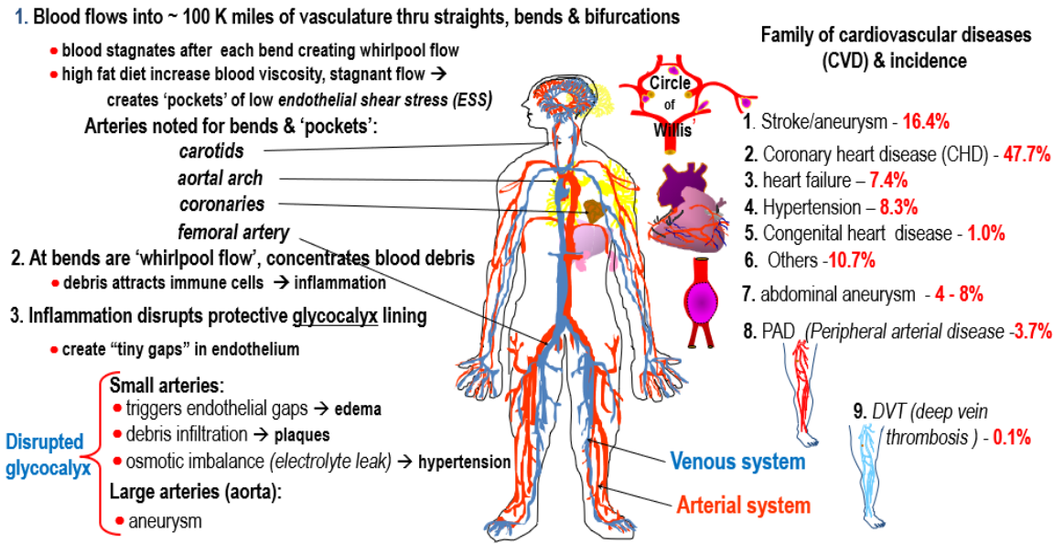

Figure 3. Family of CVD as manifested in different parts of the vascular system

1.4. Animal models for drug discovery

1.4.1. Rabbit as early model for atherosclerosis. Associating elevated level of blood cholesterol to CVD was borne out of an experiment by a Russian scientist (Ignatowski) who fed meat, eggs and milk to a rabbit and observed arterial lesions (aka, "fatty streaks", "cholesterol plaque"), which begat the "Cholesterol Hypothesis" **(93)** Rabbits, being herbivore, do not have the innate ability to metabolize meat products including fat and cholesterol, consequently they accumulate in the circulation and deposited as arterial lesions. This serendipitous experiment was misconstrued to happen also in humans, which established the consensus that dietary cholesterol leads to the development of atherosclerosis in both animals and humans. This is a flawed hypothesis because humans are naturally equipped with an enzyme, cholesterol hydroxylase (CYP7A1), that converts cholesterol to bile and prevent cholesterol accumulation. Indeed, arterial lesions in cholesterol-fed rabbits regressed when switched to low-fat chow **(94, 95).** Regardless, the rabbit became a de facto model in the study of the pathogenesis and development of human atherosclerosis. The rising incidence of heart disease in the 20s evoked the Russian "Cholesterol Hypothesis" and became the foundation of the "War on Cholesterol" to treat CVD **(96).** In 1986 the National Institutes of Health (NIH) and the American Heart Association (AHA) established the National Cholesterol Education Program (NCEP) to officially declare blood cholesterol over 200 mg/dl as a disease, which cause atherosclerosis in humans. On the other hand, carnivores like dog only develop atherosclerosis via a defective thyroid gland **(97).** Dogs with hypothyroidism have increased very low-density lipoproteins (VLDL), LDL, and HDL **(98).** A cat, another carnivore, becomes obese on carbohydrate diet **(99).** because carbohydrate is not essential for cats **(100).**

1.4.2. Gene deficient animals as models for atherosclerosis. The discovery of the LDL-R gene inspired the development of gene deletion or knock-out (KO) mice and became a default model for cholesterol buildup, which was instrumental in the discovery of the cholesterol-lowering statins. KO models include the LDL-R (*LdlR*-/-) mouse **(101)** and the apolipoprotein E (*ApoE*-/-) mouse **(102, 103).** Currently, the ApoE−/− mouse

is the model of choice in the study of human atherosclerosis and accounted for the development of more statins and PCSK9 inhibitors including Repatha and Praluent.

1.4.3. KO mouse or rabbit plaques are not the same as humans. Although KO mice and rabbits have conceptually modeled plaque formation, none of them exhibit the full characteristics seen in humans. KO mice do not reflect the human form of atherosclerosis **(104)** and thrombosis **(105)** KO mice "plaques" are superficial cholesterol lesions with no fibrous cap **(106, 107, 108),** while human plaques are subendothelial with thick fibrous cap of collagen and elastin **(109)** ***(Fig. 4).*** The fibrous cap made up of intimal smooth muscle cells and connective tissue is a healing response to encapsulate the toxic products accumulating in the necrotic core of atherosclerotic lesions **(110).**

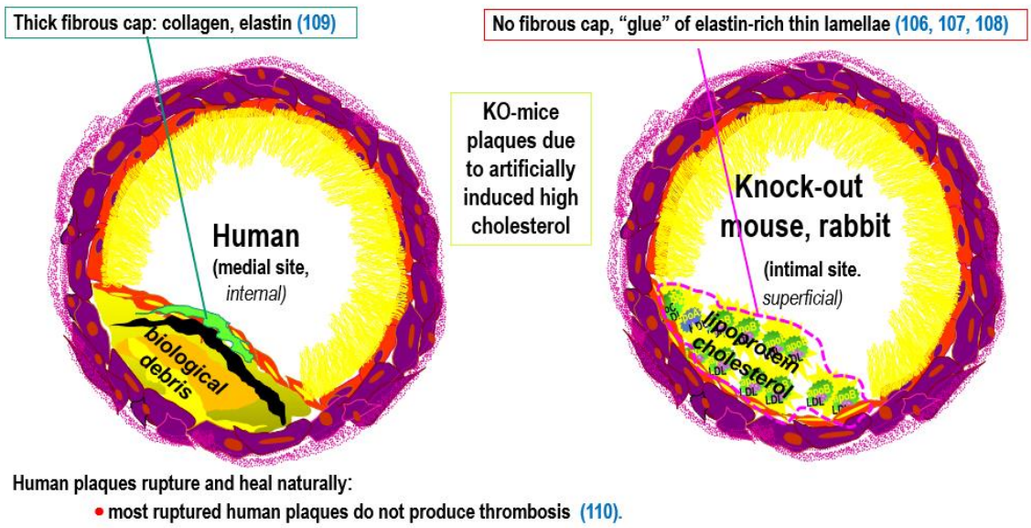

Figure 4. Plaques produced in animal models are superficial, while human plaques are subendothelial

1.4.4. 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 were described as yellow dots, visible to the unaided eye at the root of the aorta, which become more extensive at puberty and in adults, then develop to complex fibrolipid or fibrous plaque **(111).** The World Health Organization **(112)** 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 **(113).** AHA initially classified plaques into 3 types; the assumption was for type I to progress through type III **(114).** 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 ***(Table 1).***

Table 1. Three types of plaques to unify lesions from cholesterol-fed animals with clinical data

AHA's "Committee on Vascular Lesions of the Council on Arteriosclerosis" (115).

- ***to reconcile clinical and animal lesions***

Initial plaques classification: 3 basic lesions:

- **Type I: microscopically small yellow dots found in infants in the first 8 months of life (116).**
  - **» comparable to foam cells in cholesterol-fed animals: swine (117), pigeons (118), rabbits (119), monkeys (120).**
- **Type II: fatty yellow-colored streaks, stain red with Sudan III; specific to fats (triglycerides) (121).**
  - ***» readily produced in laboratory animals;***
- **Type III: characterized by pools of extracellular fat in young adults (122).**
  - ***» assume to progress from type II based on hypercholesterolemic animals* (123).**

No clear progression to symptom producing atherosclerosis,

- **developed KO mice to model plaque stability and rupture**
  - **» ApoE-/- & LDL−/− : model of stable plaques (124).**
  - **» ApoE−/−Fbn1 C1039G+/− with deleted fibrillin-1 gene (Fbn1): model of plaque rupture (125).**

1.5. Biomarkers of CVD

1.5.1. Biomarkers of CVD and drug development. Biomarker is a biological element measured and evaluated as an indicator of normal biological- or pathogenic-processes and pharmacologic responses to a therapeutic intervention **(126).** Consistent with the one-drug-one-disease paradigm, a single biomarker is selected a priori to diagnose a disease. The appeal of a single marker is its simplicity and low cost, but does not capture the xenoplexic nature of CVD, which makes a single biomarker non-specific, inaccurate, and non-sensitive.

In the clinic, diagnosis of diseases is typically surmised from symptoms, patient history, physical examination and often, one or more medical tests. However, many signs or symptoms are nonspecific. For example, cardiovascular disease, diabetes, or cancer displays multiple symptoms and therefore each of these diseases needs differential diagnosis to improve accuracy. An ideal biomarker should be diagnostic (disease presence), prognostic (measure outcome) and predictive. The rapid and correct identification of diseases is crucial and important as a guide for appropriate therapy.

Consistent with the xenoplexic nature of CVD, numerous biomarkers have been identified to be associated with this disease **(127),** but no single biomarker is yet found to be reliable in predicting or diagnosing a cardiovascular event, thus a trend towards a panel **(128).** In this study, 4 biomarkers were identified from GCX shedding, including heparan sulfate (HS), hyaluronan synthase-1 (HAS-1), syndecan-1 (SDC-1) and plasminogen activator inhibitor–1 (PAI-1) **(129).** Each of these molecules have been used in the clinic as single biomarkers ***(Table 2)*** but will be used in this study as a 4-panel diagnostic "fingerprint" to monitor therapeutic efficacy.

Table 2. Biomarker levels in blood of diseased and healthy patients.

Biomarker	Disease type	Level		References
		healthy	*diseased*	
Syndecan-1 (SDC1)				
	Myeloma	128 units/mL	1170 units/mL	(130)
	Lung cancer (SCLC)	16 ng/ml	44 ng/ml	(131)
	Heart failure	1.19 ng/ml	4.14 ng/ml	(132)
	Inflammatory bowel disease (IBD)	21.2 ng/mL	29.5 ng/mL	(133)
	Acute decompensated HF (ADHF)	91.4 ng/ml	133.7 ng/ml.	(134)
	Chronic heart failure	5.7 ng/mL	22.5 ng/mL	(135)
	Acute coronary syndrome (ACS)	42 ng/ml	77 ng/ml	(136)
	Global and regional ischemia	1.2 ug/dL	50.4 1.2 ug/dL	(137)
	Preeclampsia	28 ng/mL	218 ng/mL	(138)
	Renal failure	27.5 ng/ml	111.0 ng/ml	(139)
	Ischemia	1.2 µg/dL	1.38ug/dL	(140)
	Hemorrhagic Shock	27 ng/ml).	554 ng/ml	(141)
Heparan sulfate (HS)				
	Dengue fever (hemorrhagic	16.78 ng/ml	108.55 ng/ml	(142)
	Ischemia	590 ug/dL	5,900 ug/dL	(143)
	Mucopolysaccharidoses (MPS)	12.4 ug/ml	24.4 ug/ml	(144)
	Hypertension (pre-eclampsia)	60.5 mg/l;	123.1 mg/l	(145)
Hyaluronan synthase (HAS-1)				
	Arthritis	29.1 ng/ml	37.4 ng/ml	(146)
	Stroke	170.4 ng/ml	219.7 ng/ml	(142)
	Liver disease (alcoholic cirrhosis)	0.03 ng/ml	1.08 ng/ml	(147)
	liver cirrhosis	30-40 ug/L	100 – 300 ug/L	(148)
	Hepatitis (chronic)	117.86 ng/ml	575.93 ng/ml	(149)
	Fibrosis	16 µg/l,	121 µg/l,	(150)
	malignant pleural mesothelioma	≤49 µg/ml	>100 µg/ml	(151)
	Septicemia	11 ug/L	344 ug/L:	(152)
	acute stroke patients	170.4 ng/ml.	219.7 ng/ml	(142)
	Dengue fever	100.11 ng/ml	935.91 ng/ml	(153)
Plasminogen activator inhibitor 1 (PAI-1)				
	ischemic stroke:	11.8 IU.	17.2 IU	(154)
	Acute Stroke	23.6 ng/ml	45.2 ng/ml	(155).
	Chronic angina pectoris	9.6 U/ml)	17.5 U/ml)	(156)
	Acute myocardial infarction	10.1 ng/ml	16.3 ng/ml	(157)
	Angina	40.0 ng/mL	95.4 ng/mL	(158)
	Coronary arterial disease (CAD)	2.97 ng/ml	5.26 ng/ml	(159)
	Acute myocardial infarction	9.35 ng/ml	44.02 ng/ml	(160)
	Hypertension:	32.1 ng/mL	39.8 ng/mL	(161)
	Coronary Artery Disease	2.4 ng/ml	8.8 ng/ml	(162)
	ST-elev myocardial infarction (STEMI)	16.1 U/mL	21.9 U/mL	(163)
	Glioma	2.5 ng/ml	14.7 ng/ml	(164)
	Acute Myocardial Infarction	10.0 ug/L	15.1 µg/L	(165)

Indeed, monitoring GCX shedding promises to be an important diagnostic tool for CVD in clinical settings **(166).** However, the current GCX diagnostic techniques involve direct and indirect microscopy **(167),** which are impractical.

2. OBJECTIVES

KO mice and rabbits are models of atherosclerosis focusing on cholesterol plaques, and do not reflect the multiple nature of cardiovascular disease (CVD). One-drug-one-target paradigm has produced the statins but are at best palliative. CVD is a xenoplexic disease and this study aims to address its complex etiology by combo-compound therapy.

Evaluation of therapeutic effect will use a wild mouse made atherosclerotic by xenobiotics including high fat diet and an environmental pollutant **(129).** The objectives of this study are:

1. Construct a virtual etiology of CVD, particularly a thromboembolic cascade, and identify “druggable” targets.
2. Synthesize new chemical entities (NCEs) to target specific sites in the thromboembolic cascade.
3. test these NCEs in combo to address in toto the xenoplexic nature of the disease.
4. Confirm plaque formation with histopathology and create a 4-panel biomarker as a surrogate diagnostic ‘fingerprint’.
5. Evaluate drug effectiveness in both preventive and curative mode

3. MATERIALS AND METHODS

3.1. Mice.

A wild type C57BL/6 mouse, non-genetically altered mouse is used as an atherosclerosis model **(129)** for this study. Briefly, arterial plaque formation was achieved by treating wild c57Bl/6 with high fat and PCB,

which produced plaques as confirmed by histopathology. This mouse model was used in this study and herein called the Tunac Arterial Plaque (TAP) mouse model. 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). Ethics regulation of laboratory animals: This study was conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

3.2. Treatments.

It is well known that environmental pollutants including the lipophilic persistent organic pollutants (POPs), such as polychlorobiphenyls (PCBs), accumulate in fatty tissues **(168).** 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 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 induce inflammation in the adipose tissues **(169).** For this study 3,3',4,4'-Tetrachlorobiphenyl (PCB-77) 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.

Compounds tested for therapeutic activities were suspended in 8 ml carboxymethylcellulose and 0.2 ml/mouse and delivered by gavage with a dose of 3.1 mg/kg according to the treatment schedule ***(Table 3).***

Table 3. Experimental setup showing treatment schedule to reflect preventive and curative modes.

DIO (high fat diet); A-L (3-NCE combos); PCB (polychlorobiphenyl); sac (harvest)

	Group	Diet	Day 1	2	3	4	5	6	7	8	9	11	18
Preventive	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

3.3. Enzyme-linked immunosorbent assay (ELISA).

ELISA is a blood test that detects an antigen, and in this study the chosen antigens were debris or detritus from disrupted GCX. Previous work (129) showed high correlation of plaque formation to elevated ELISA

values on 4 biomarkers including Heparan Sulfate (HS), Hyaluronan Synthase 1 (HAS-1), Syndecan 1 (SDC1), and total Plasminogen Activation Inhibitor-1 (PAI-1). These biomarkers are used as a 4-panel diagnostic 'fingerprint' to evaluate the curative or preventive effect of the therapeutic compounds: preventive (PCB added day 7 & 9 to 16-day old mice on fat diet, then therapeutics 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).

HS and HAS-1 kits were obtained from Antibodies-Online (Limerick, PA) SDC-1 from USCN (Houston, Texas) and PAI-1 from Molecular Innovations (Novi, MI). 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. All tests were performed on plasma, diluted to fall within the standard curve, and carried out according to the manufacturer's instructions

3.4 Statistical analysis

Data presented as the standard error of the mean (SEM) and ANOVA used for analysis. A *P* value <0.05 was considered significant ***(Table 4)***

Table 4. Statistics for biomarker assays (p values)

Protocol	Combo	Group	HAS-1	HS	PAI-1	SDC-I
PREVENTIVE	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	.001	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	.118	.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

3.5. 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.

4. RESULTS

***4.1. 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 therapeutic compounds.

4.2. Drug targets and synthesized compounds.

Understanding the disease cascade and recognizing dysfunctional sites is key to designing drugs. The main pathogenetic mechanism in CVD is thromboembolism, thus a conceptual thromboembolic cascade was constructed and identified 5 druggable targets ***(Fig. 5).***

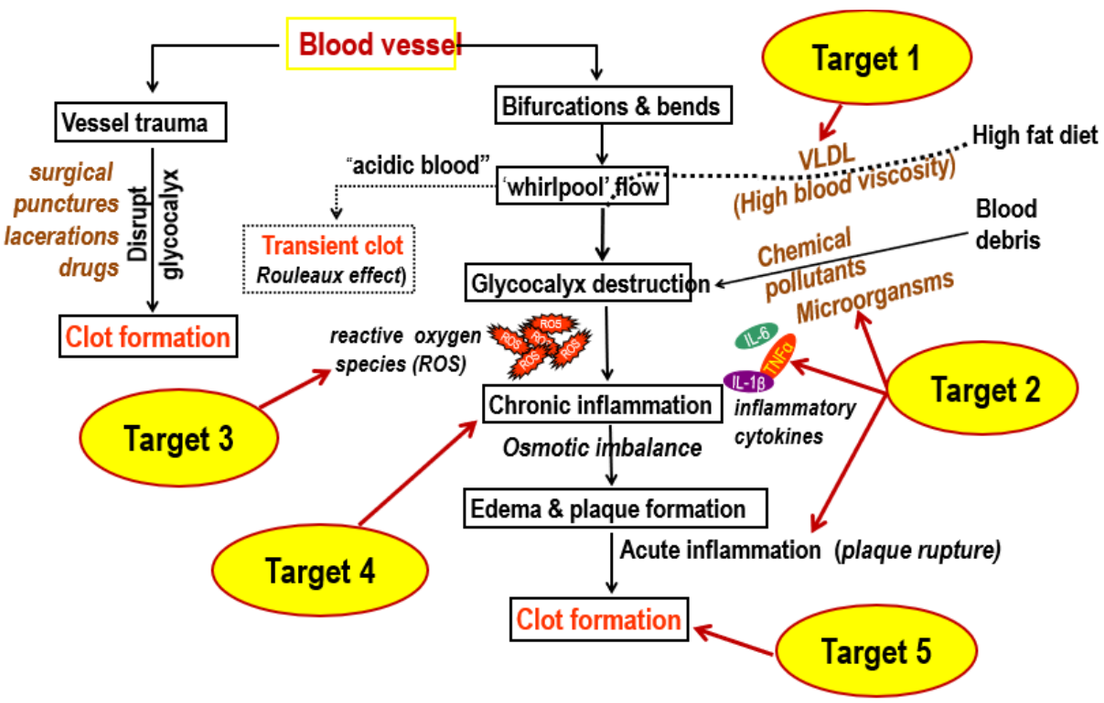

Figure 5. A virtual thromboembolic cascade and possible "druggable" sites

4.3. Synthesis of compounds for druggable targets.

8 new chemical entities (NCEs) were rationally synthesized for the corresponding targets: FTX-214, -216, --218, -219, -224, -226, -229, -230. Synthesis of these NCEs was based on empirical knowledge of active drug scaffolds and mode of action to address the indicated targets. Since no single NCE could address in toto the thromboembolic cascade, a combo-compound therapy was the best option. Thus, an abbreviated 3-factor permutation combination was carried out on the 8 NCEs, which resulted in twelve 3-NCE combos

(A-L), hereafter called 'combo compound or combo'. The combos were formulated at a fixed dose 1:1:1 ratio. The structures of the NCEs designated as FTX and the 3-NCE combos are shown in ***Fig. 6.***

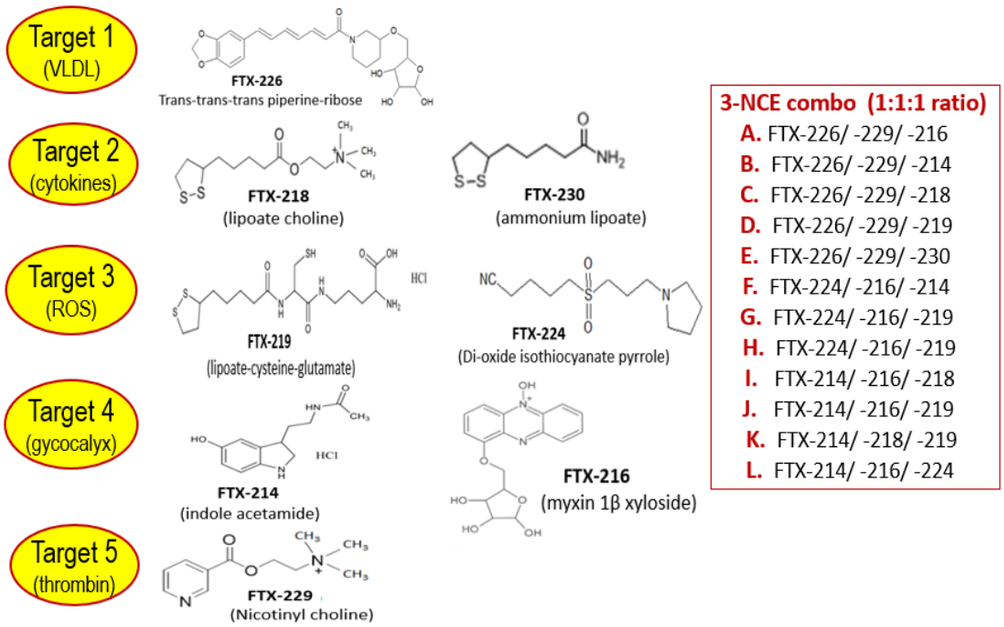

Figure 6. Structures of 8 new chemical entities (NCEs), targets, and components of 3-NCE combos (A-L)

4.4. ELISA values.

Statistical analyses were carried out to determine the correlation of ELISA data and the preventive or curative effect of the combos. A small p-value (typically ≤ 0.05) indicates strong correlation; a large p-value (> 0.05) indicates weak relationship ***(Table 4).***

Table 4. Statistics for biomarker assays (p values)

Protocol	Combo	Group	HAS-1	HS	PAI-1	SDC-I
PREVENTIVE	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	.008	.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	.118	.989	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

There are three untreated controls: 'normal' (regular diet), 'DIO' (high-fat diet) and 'none' (high-fat diet + PCB). The normal and DIO reflect the baseline level of biomarkers indicative of normal turnover or shedding of these GCX components. Taking the average ELISA values of these 2 controls, the turnover rate is as follows: PAI-1 (2,096 pg/ml) > HS (106 pg/ml) > SDC-1 (66 pg/ml) > HAS (19 pg/ml) ***(Fig 7).***

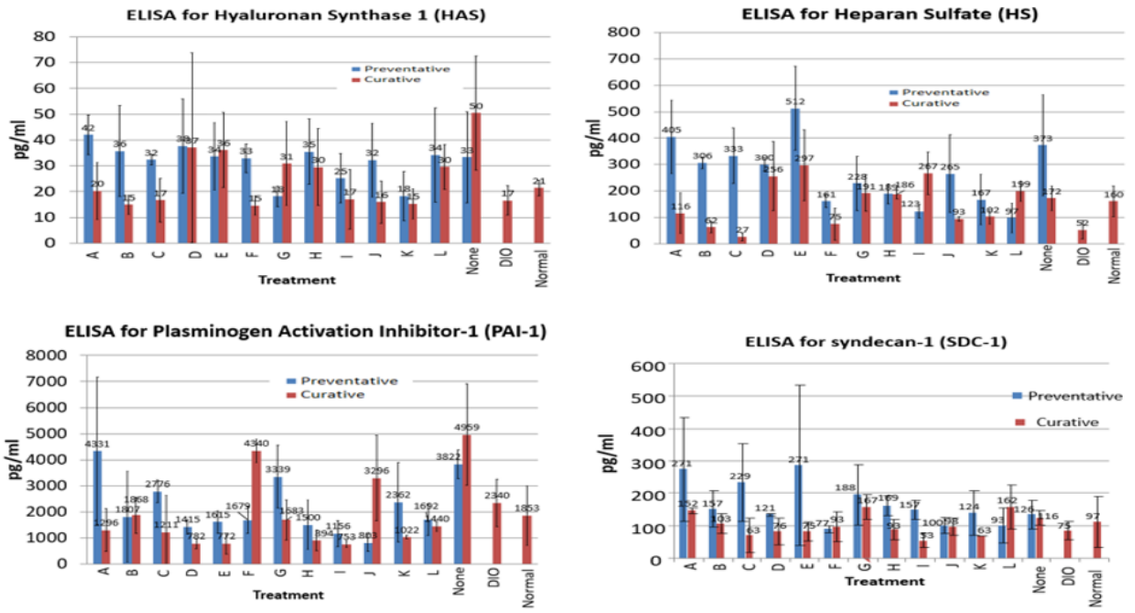

Figure 7. Response profiles of the 4 biomarker to 3-NCE combo treatment

The 3rd control are mice fed with high fat diet and gavaged with the PCB pollutant. This control group was subdivided in two to provide baseline reference point for "preventive" as well as "curative". Thus, ELISA biomarker level of a combo-treated mouse is subtracted from the corresponding control to give the net therapeutic activity. In this regard, the therapeutic activities of the combo compound are reflected by the reduction in ELISA values:

4.4.1. Hyaluronan synthase (HAS-1). Reduced levels were observed in mice treated with Combos G, I and K in the Preventative Protocol, and Combos A, B, C, F, I, J, 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 $p<0.002$ to $p<0.01$.

4.4.2. Heparan sulfate (HS). Reductions in HS levels were observed in mice treated with Combos F, G, H, I, K and L in the Preventive 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 $p<0.001$.

4.4.3. Total Plasminogen Activation Inhibitor-1 (PAI-1). Reductions in PAI-1 levels were observed in mice treated with Combos B, D, E, F, H, I, J, K and L in the Preventive Protocol, and all Combos except F and J in the Curative Protocol. ANOVA statistics for the comparison of levels revealed significant changes in the Preventive Protocol, with decrease due to Combos B, D, E, F, H, I, J and L ranging from $p<0.02$ to $p<0.001$.

4.4.4 Syndecan1 (SDC1). Reductions in SDC1 levels were observed in mice treated with Combos D, F, J, and K in the Preventive Protocol, and Combos B, C, D, E, F, H, I, J, and K in the Curative Protocol.

4.5. Summary of therapeutic activities:

4.5.1. Combo preventive and curative activity ratings. The therapeutic effect of the 12 combos (A-L) vs the 4-panel biomarker is presented in numerical format ***(Table 5)*** as well as graphics ***(Fig 8).***

Table 5. Comparative activities of the 12 (A-L) 3-NCE combos showing inactive (red) and active values per ELISA levels.

3-NCE combo (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
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. 214/216/218	25	17	123	*267*	1156	753	*157*	53
J. 214/216/219	32	16	265	93	803	3296	100	98
K. 214/218/219	18	15	167	102	2362	1022	124	63
L. 214/216/224	*34*	30	97	*199*	1692	1440	93	*162*
Control *(no combo)*	33	50	373	172	3822	4959	126	116

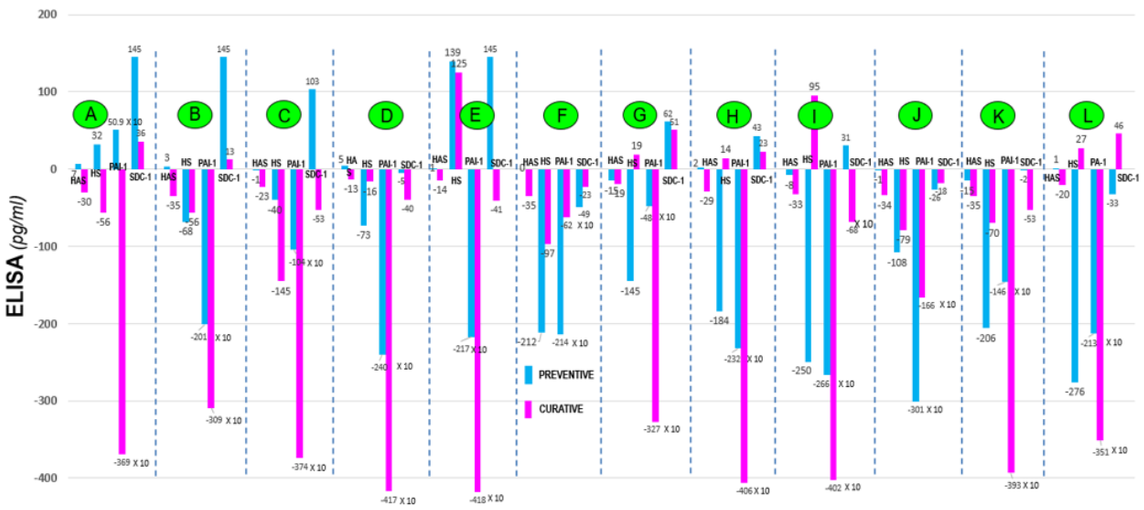

Figure 8. Graphical presentation of the preventive and/or curative activities of the 12 (A-L) 3-NCE combos per 4-panel ELISA levels. Combos D, F, J and K show both preventive and curative activities

A combo that did not reduce biomarker level (red) is considered inactive ***(Table 5),*** which is represented as "positive" bars in ***Fig. 8***. Combos D, F, J and K were preventive and curative. Combo A was predominantly curative (except vs SDC-1); on the other hand, there was no combo found to be selectively preventive. Combo K was chosen for further evaluation because of its overall superior profile (K >J > F > D) and hereby designated Embotricin™

4.5.2. NCE activity rating. Individual NCEs showed inherent activities with the following order based on frequency of appearance in the active combo. For example, the individual components of combos K, J, F

and D, which were curative/preventive of plaques are ranked as follows (frequency): FTX-214 (3) > FTX-219 (3) > FTX-216 (2) > FTX-218 (1) = FTX-224 (1) = FTX-226 (1) = FTX-229 (1). The targets in order of significance follows: GCX repair (FTX-214, -216) = ROS (FTX-219, - 224) > inflammation (FTX-218) = VLDL (FTX-226) = thrombin (FTX-229).

4.6. Histopathology

4.6.1. Plaques. While the curative and or preventive effects of the combos were evaluated by ELISA, presence of plaque was confirmed by histopathology, particularly in the brachiocephalic artery. Typical histopathology shows plaque formation in the control (no NCE treatment) while no plaques in the combo treatment. For example, the control animals (High fat diet, PCB) showed plaques typically with a fibrous material loosely attached to the surface of the arterial wall ***(Fig. 9A, 9B).*** In contrast, the combo treated animals including Embotricin™ exhibited the typical features of a normal arterial wall ***(Fig. 9C).***

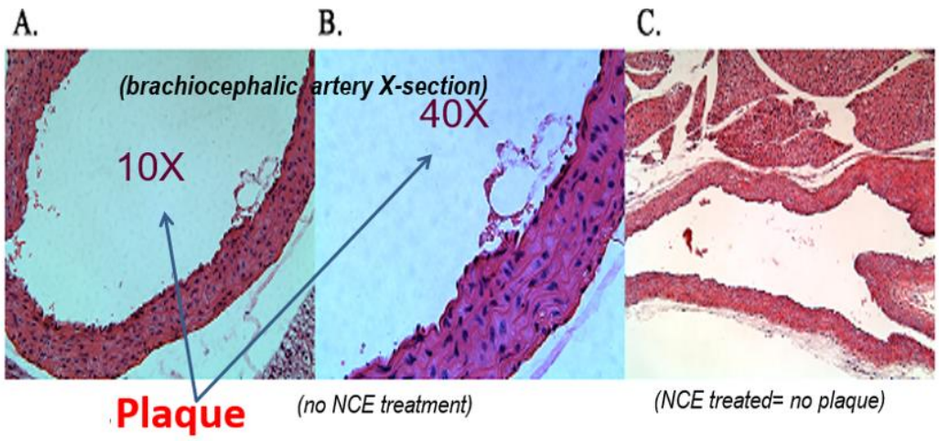

Figure 9. A plaque produced in the TAP mouse located in the subendothelial region with fibrous cap similar

Other histopathological features in the control animals showed various lesion stages including lipid deposits, fatty streaks and eventually a well-defined plaque with fibrous cap ***(Fig. 10c).***

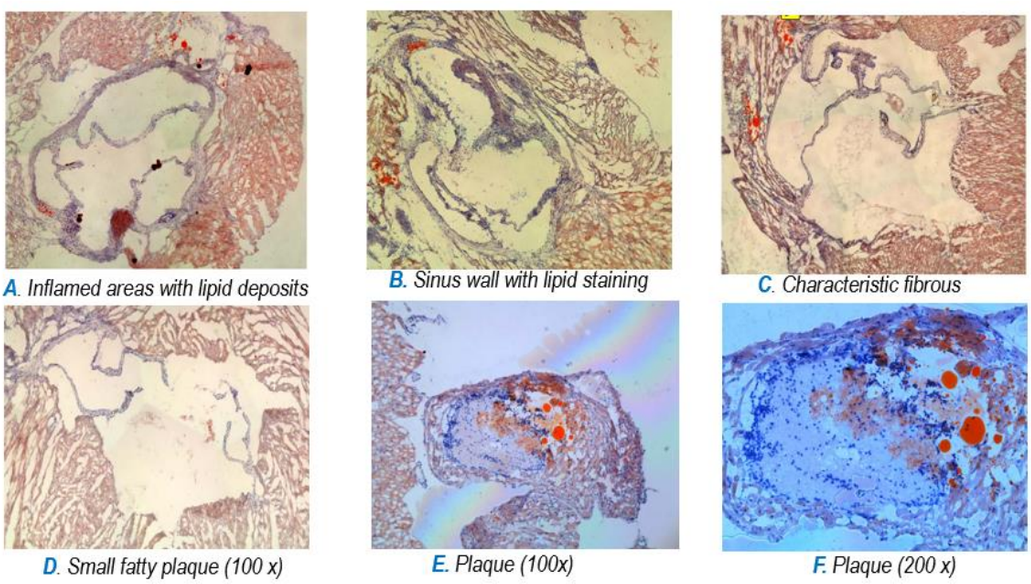

Figure 10. Stages of lesion development in the TAP mouse model including lipid deposits fatty streaks and eventually a well-defined fibrous cap.

5. DISCUSSION

5.1. Drug discovery paradigms.

The traditional drug development strategy is based on the 'one gene-one disease' paradigm, searching for a target gene to develop a 'magic–bullet'. This paradigm assumes that a disease is caused by dysfunctional gene, which inspired the mapping of the human genome to identify mutant genes **(170)** and the evolution of the 4 "omics", including genomics (DNA, RNA), proteomics (proteins), metabolomics (metabolites) and glycomics (sugars) to classify target genes **(171).** A gene is the basic unit of heredity defined by a specific nucleotide sequence, which is studied under genetics and epigenetics. While genetics study genes that alter genetic material to control functions, epigenetics study modification of gene expression due to acquired "ornamentations" including DNA methylation, histone modification, RNA acetylation, etc**. (172).** These epigenic "ornamentations" are triggered by extraneous factors, stress, and drugs **(173).** For example, > 90% of disease risks are epigenetic in nature **(174),** which accounts for sporadic cancer **(175)** and CVD **(176).**

The G protein-coupled receptor (GPCR) represent the largest protein family encoded by the human genome and the most prominent gene target for therapeutic agents **(177).** There are 2350 epigenetic "mutations" attributed to GPCRs from which over 66 human diseases have been identified **(178)** and over 160 GPCR genes are being evaluated as drug targets in mice **(179).** GCPRs are nested in the GCX of the cell membrane, which binds extracellular substances and transmits signals to an intracellular molecule called a G protein (guanine nucleotide-binding protein) **(180).** GCX disruption affects the GPCRs downstream signaling pathways and the enzymes associated with these downstream pathways are the targets of drug development **(181).** These are epigenetics in nature and could be temporary, occur during a lifetime, or heritable **(182).** In this regard, genetics and epigenetics crisscross each other complicating the understanding of disease etiology **(183).** Even FH, a genetic variant in LDLR, APOB, or PCSK9, now reveals major DNA methylation patterns characteristic of epigenetics **(184).** Also, effect of fatty acid diet is now found to have epigenetic mechanism on CVD and other chronic diseases **(185).**

At the molecular level, epigenetic changes involve the "ornamentations" within the nucleic acids and histone proteins. These "ornamentations" consist of three families of proteins including "writers", "reader" and "erasers", which are druggable targets. For example, "writers" are enzymes that add ornamentation, "readers" control binding of such enzymes and "erasers" regulates reversibility of both writers and readers **(186).** Drugs that target ornamentations are called epidrugs **(187)** with a global market of $7.50 billion in 2019 and grows to $31.64 billion by 2027 **(188, 189, 190)**, which includes inhibitors of DNA methyltransferase, histone deacetylase (HDAC), histone methyltransferase, lysine demethylase. As monotherapies these inhibitors modulate downstream effects of these enzymes, but they do not treat the disease in toto **(191).** Identifying and targeting the multitude GPCR genes is an iteration of the 'one gene-one enzyme/disease' drug discovery platform, which has not delivered the promised "magic bullet".

Key to the success of a drug development program is understanding the upstream disease etiology. This study recognizes that vascular diseases including CVD are triggered by extraneous (xeno) factors, which is of multiple (plexic) origin including environment, lifestyle, and drug usage, herein called xenoplexic

diseases. Xenoplexic diseases are pathologies resulting from upstream cell disruptions and if left unrepaired becomes a downstream chronic pathology manifested in different symptoms. In this regard the complicit etiology of xenoplexic diseases is best treated with combo drugs, while the monotherapy platform targeting symptoms is at best palliative and not curative. CVD is an example of xenoplexic disease as illustrated in ***Fig. 11.***

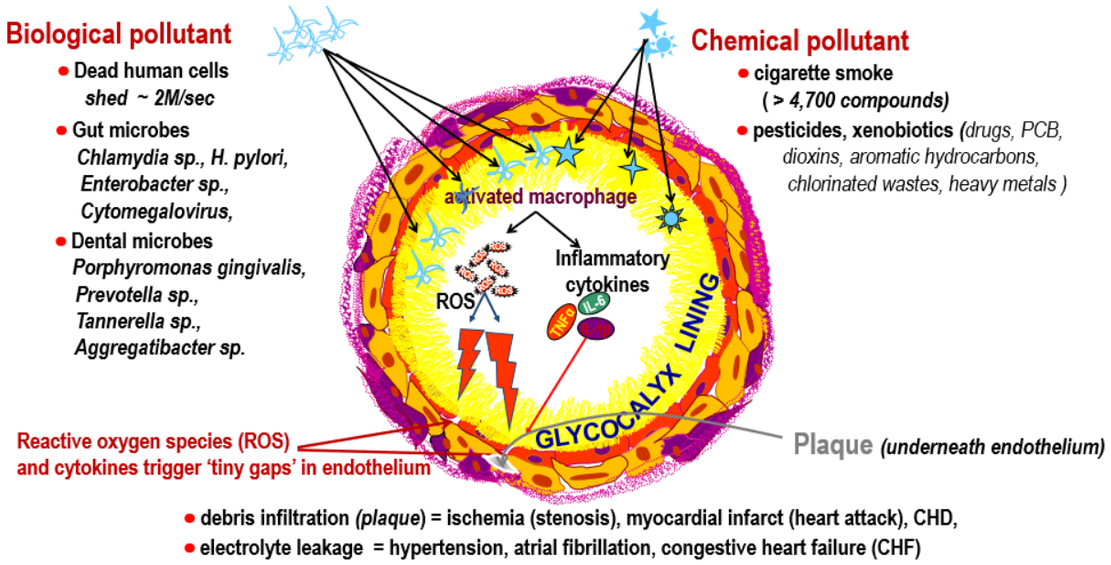

Figure 11. Biological and chemical pollutants in the arterial bends trigger inflammation, tiny endothelial gaps creating electrolyte leakage (hypertension) and debris infiltration (plaque)

***5.2. Combo drug therapy*.**

The one-gene one enzyme or the polypharmacology approach **(192)** to treating CVD has failed. Targeting a single step along a given pathway or even targeting a single pathway does not effectively treat a complex disease like CVD. Developing combinations simultaneously, rather than as individual entities is a more effective approach to treat disease progression typical of a xenoplexic disease.

***5.3. Mouse and humans**:*

Mouse and humans share close genomic homologies, whereby only less than 1% of the wild type C57BL/6 mouse, genes do not have any detectable homologue in the human genome **(193).** Mice are naturally resistant to atherosclerosis but produce arterial lesions when fed with high-fat/high-cholesterol diets supplemented with cholic acid (CA) **(194).** Several studies have varied the ratios of fat, cholesterol and cholic acid to obtain persistent hypercholesterolemia of > 300mg/dL cholesterol, which account for cholesterol lesions; but are not typical or predictive in humans **(195).** KO mice model of hypercholesterolemia also produced fatty streaks not comparable to the human plaques produced on the high fat "Western" diet **(196).** This study used a wild mouse exposed to the xenoplexic factors as humans and, indeed, produced plaques with fibrous caps like humans.

5.4. Biomarkers of CVD and drug development.

A biomarker can be a gene, a gene mutation, protein, other molecule, or clinical measurement that indicates a given disease state. Single markers, while clinically appealing due to simplicity and low cost, do not capture the complicit nature of xenoplexic disease, which makes them inaccurate, insensitive, and non-specific. To improve the prediction of death from cardiovascular causes, a combination of biomarkers

including troponin, natriuretic peptide, cystatin C, C-reactive protein was proposed **(197).** In this study, the components and sequela of GCX disruption were chosen as a diagnostic panel including: Hyaluronan synthase (HAS-1), Heparan sulfate (HS). Total Plasminogen Activation Inhibitor-1 (PAI-1) and Syndecan1 (SDC1).

It appears that as the GCX is disrupted the endothelium releases an embedded protein called plasminogen activator inhibitor-1 (PAI-1); this protein mainly produced by the endothelium suppress fibrinolysis or hemorrhaging; heparan sulfate is found in the outermost layer of the protective glycocalyx and the first to be shed when the shield is compromised. As disruption advances, syndecan -1 (Syn-1), which maintain cytoskeletal integrity is shed, followed by hyaluronan, a glyocosaminoglycan that abuts the heparan sulfate. These biomarkers have been used in the clinic as single markers with limited success, but the failure of individual markers is that in a xenoplexic disease a mixture of these biomarkers is present in the milieu at any given time in different levels. Thus, picking one biomarker does not reflect the dynamic process, which lowers its diagnostic or predictive capacity. Thus, a panel of biomarkers used as a “fingerprint” is a more appropriate diagnostic tool for xenoplexic diseases like CVD.

5.5. The significance of this study. The integrity of the GCX is maintained by a balanced synthesis and shedding of its component parts. While excess shedding is associated with cardiovascular risks, it is possible to stop excess shedding and maintain a homeostatic balance. Embotricin™ restores the integrity of the GCX because of its combo compound components as evidenced by plaque prevention and reversal. Death of patients with CHD is due to disruption of an atherosclerotic plaque with subsequent complete or partial vascular thrombosis **(198).** The presence of plaque is the hallmark of coronary atherosclerosis and a prerequisite for increased acute coronary events **(199)** and risk of death increases with the extent of plaques **(200).** Even though most plaque disruptions do not lead to acute coronary events, some do and the more plaques that are present the more plaque disruptions occur and the greater the probability that one of them triggers vascular thrombosis **(201).** The robust preclinical data of Embotricin™ effectively targeting plaque may well be the first curative (anti-embolic™ drug against CVD with the same impact as penicillin to infectious disease.

5.6. Conclusion. The proposed mode of action (MOA) of Embotricin™ is a synergy of its component compounds ***(Fig 12):*** FTX-214 upregulate aryl hydrocarbon receptor (AhR) and triggers multigene expressions including xenobiotic metabolizing enzymes [e.g., cytochrome P450 (CYP) 1A1, B1], extracellular matrix synthesis, cholesterol biosynthesis and cell signaling at the cell membrane **(202).** AhR deficiency triggers inflammation and disrupts matrix biology resulting in diseases **(203).** FTX-218 binds with the oxidized Nrf2-Keap-1 complex and release NRF2. Nrf2 subsequently transcribe the genes to produce a myriad of antioxidant enzymes including, Glutathione transferase (GST), Glutathione synthetase (GS), Glutathione peroxidase (GPx), Superoxide dismutase (SOD), Catalase (CAT), to protect cell from free radicals (R•) and other reactive oxygen species (ROS) **(204, 205).**

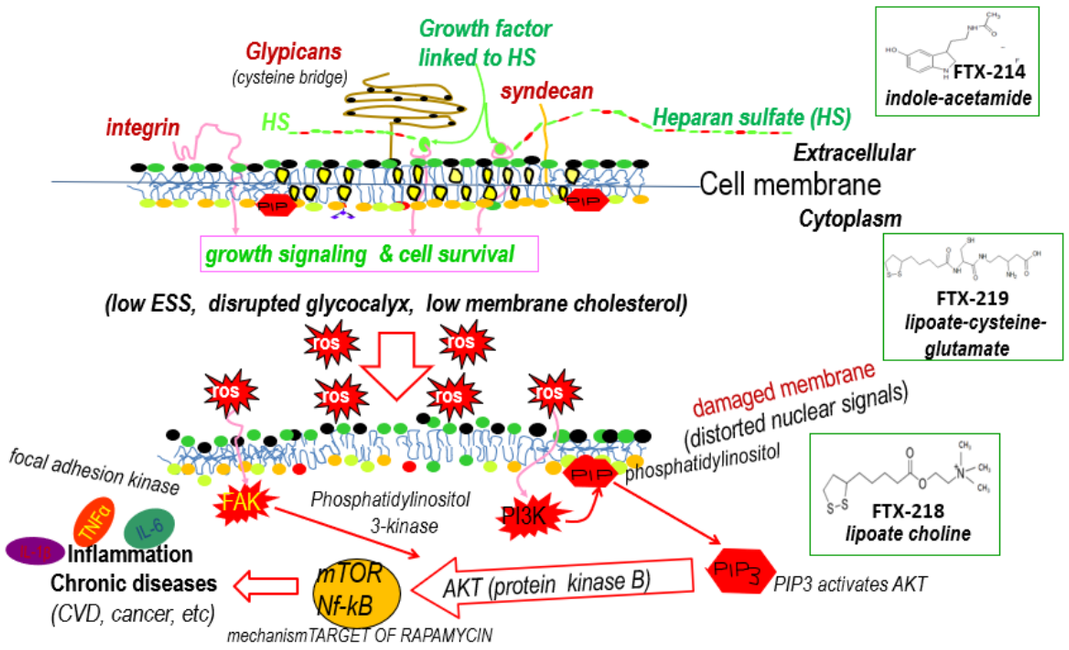

Figure 12. Proposed molecular mode of action of the three NCE components of Embotricin™

FTX-219 inactivates the nuclear factor kappa B (NF-κB), which is a crucial to various biological processes, including immune response, inflammation, cell growth and survival, and development **(206, 207),** NF-kB is activated during oxidative stress **(208, 209)** triggering inflammatory responses **(210).**

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CONFLICTS OF INTEREST

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