Introducing Arterez
"Glycalyx Detritus Fingerprint™ Technology"

Dr. J. B. Tunac

Glycocalyx (GCX) disruption is at the root of multiple chronic diseases.

GCX is the first line of defense to proper blood flow

Body cells are organized into four tissues including epithelium nervous, muscle and connective tissue. Epithelium covers body surfaces, lines internal closed cavities including glands, body tubes and the vascular system. Epithelial tissue (a) protects underlying tissues from radiation, desiccation, toxins, pathogens, and physical trauma, (b) regulates exchange of chemicals between tissues and a body cavity and (c) secretes hormones into the blood vascular system, providing sensation. Endothelial cells line the internal surface of the circulatory system including the lumen of the arteries, veins, lymphatic vessels, blood capillaries and cavities of the heart. Yet another layer on top of the endothelium is the glycocalyx, which provides the first line of protection from physical, chemical, and biological wear (Fig. 1)

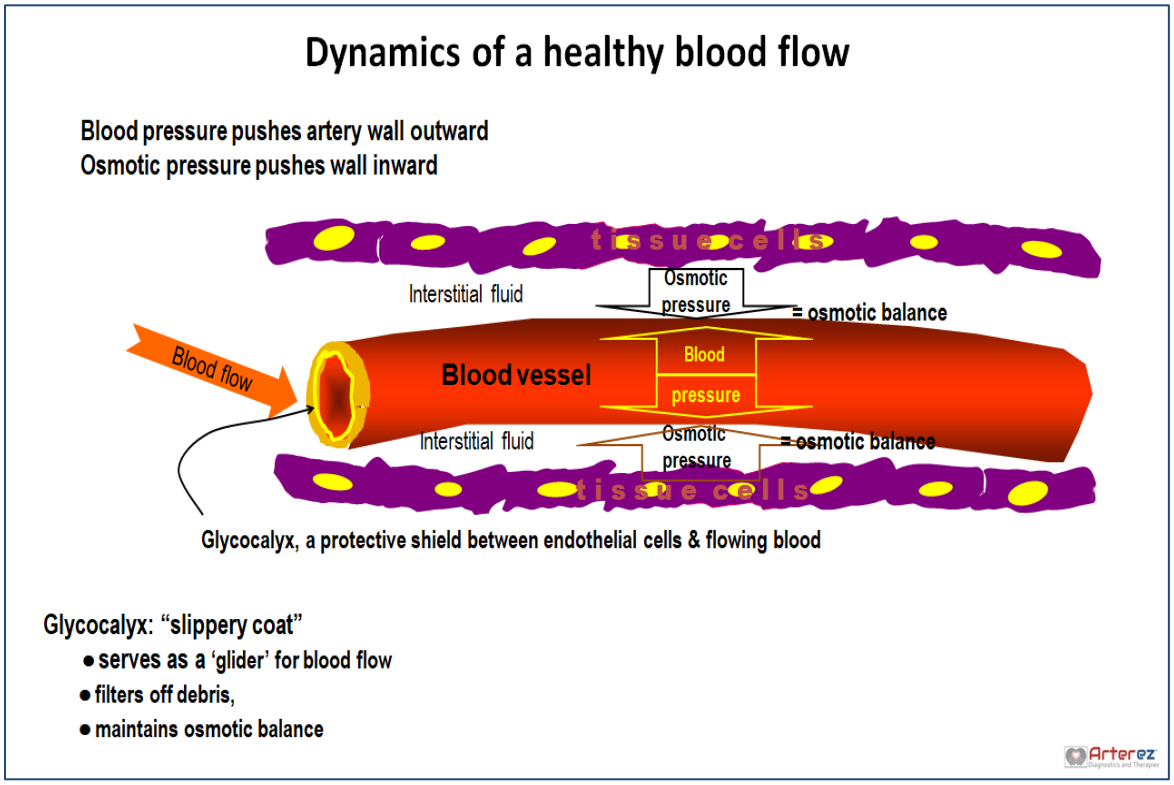

Figure 1. Blood vessel lined with a 'slippery lining', the glycocalyx which promotes healthy blood flow.

GCX consists of multiple interwoven components to maintain integrity of the endothelial

GCX is a fuzz-like carbohydrate-rich coat that projects out and covers the membrane of endothelial cells, which filters off cell debris and prevents adhesion of coagulatory and inflammatory cells to the vascular endothelial lining. Other critical function of the GCX include: 1) transmits fluid shearing forces to the cytoskeleton of endothelial cells and stimulates the production of nitric oxide, vital to controlling blood flow and blood pressure, 2) regulates the supply of nutrients and oxygen, 3) removes waste and carbon dioxide, and, 4) maintains capillary integrity, preventing loss of fluid through leakage (Fig. 2)

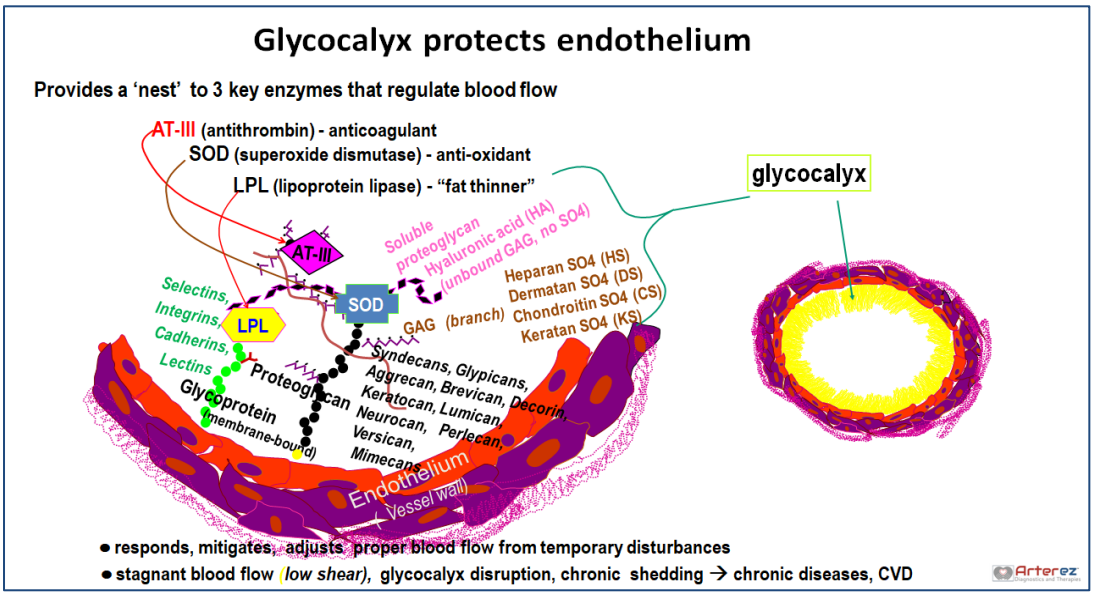

Figure 2. Anatomy of the protective endothelial glycocalyx showing glycoprotein and proteoglycan components, including nested proteins (AT-III, SOD, and LPL)

GCX is connected to the endothelial cell via several glycoprotein and proteoglycan backbone molecules [2007.Pflugers Arch. 454(3):345–359.]. The glycoproteins are protein-glycan conjugates (2006. J Intern Med. 259(4):339–350), which are adhesion molecules that contribute to pathological state (2014. Anaesthesia. 69(7):777–784). The three families of adhesive molecules include the selectin family, the integrin family, and immunoglobulin superfamily (2006. J Intern Med. 259(4):339–350].

Disruption of GCX triggers epithelial and vascular diseases including CVD

The GCX is an extracellular matrix that covers the luminal surface of the vascular system. This structure is not just a barrier for vascular permeability but contributes to various functions including signal sensing and transmission to the endothelium. Thus, pathological changes to this structure are involved in the development of various diseases. (Fig. 3)

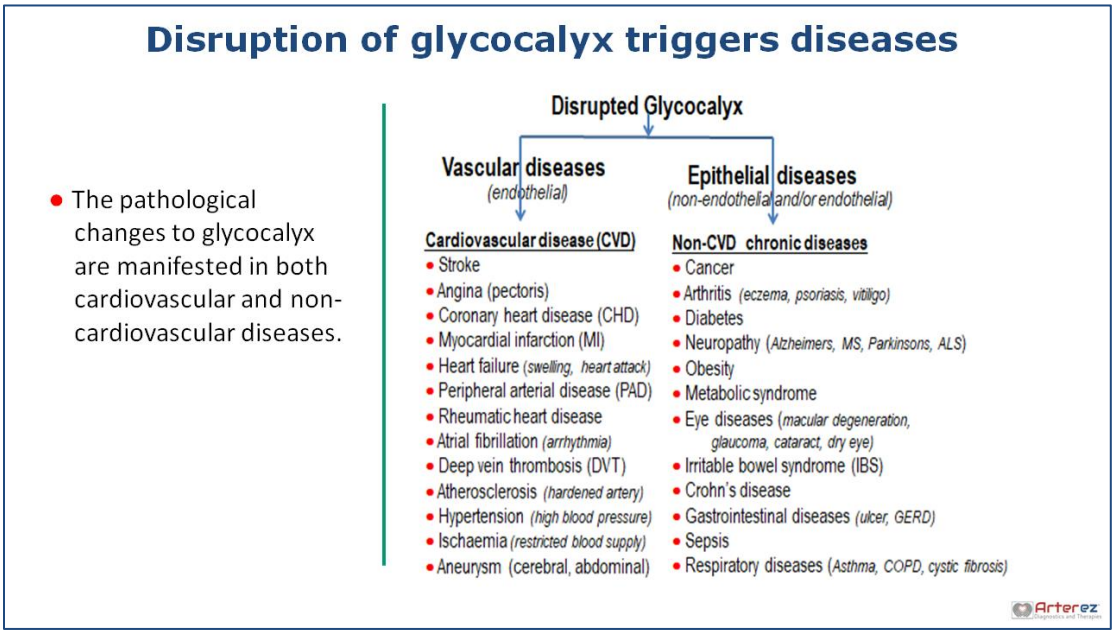

Figure 3. Disruption of the GCX accounts for a number of vascular-related pathophysiologies including CVD

The following are a sampling of recent reviews supporting GCX disruption as the root cause of a number of pathologies and thus basis for a diagnostics and therapeutic target:

https://journals.lww.com/cmj/Fulltext/2019/04200/Endothelial_glycocalyx_as_a_potential_theriapeutic.11.aspx
Endothelial glycocalyx as a potential therapeutic target in organ injuries

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6337861/
The glycocalyx: a novel diagnostic and therapeutic target in sepsis

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6262336/
Shared Features of Endothelial Dysfunction between Sepsis and Its Preceding Risk Factors (Aging and Chronic Disease)

https://jintensivecare.biomedcentral.com/articles/10.1186/s40560-016-0182-z
Glycocalyx and its involvement in clinical pathophysiologies

https://academic.oup.com/cardiovascres/article/87/2/300/446026
Therapeutic strategies targeting the endothelial glycocalyx: acute deficits, but great potential

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5681608/
Glycocalyx in Atherosclerosis-Relevant Endothelium Function and as a Therapeutic Target

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6475045/
Degradation of Glycocalyx and Multiple Manifestations of Endothelial Dysfunction Coincide in the Early Phase of Endothelial Dysfunction Before Atherosclerotic Plaque Development in Apolipoprotein E/Low-Density Lipoprotein Receptor-Deficient Mice

Utilizing fingerprints as identification and diagnostic tools

- Fingerprinting involves a multicomponent set of parameters, and therefore is a much more accurate system for identification or diagnosis.
- The components of the classic fingerprint are the physical contour of fingertips, while the DNA-fingerprint includes genetic components, both accurate for identifying individuals in forensics, paternity, etc.
- There is no fingerprint as yet for diseases. The Glycalyx Detritus Fingerprint™ is the first analytical tool to diagnose diseases, a new era in healthcare to assist in targeted treatments.
- The idea of fingerprinting started in primitive times when man used to hunt for food with the help of animal's footprints. Since, the science of 'fingerprinting' evolved as an identification system using a set of parameters:

1. **Physical parameter** - 1858: Sir William Herschel (UK) first used prints from fingertips to identify criminals, which swiftly developed into a cornerstone of forensic science of "fingerprinting". No two people have exactly the same fingerprints. This uniqueness allows fingerprints to be used for background checks (identity, employment, criminology) and biometrics security (access to secured areas). Fingerprint is the impression found when an inked finger is pressed onto paper leaving friction ridges (raised) and furrows (recessed) on the pad. Friction ridge patterns are grouped into three distinct types: loops (60%), whorls (35%) and arches (5%), which are the basic patterns (Fig 4)

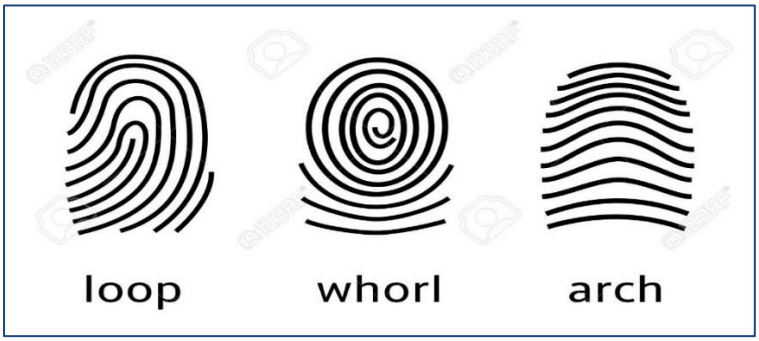

Figure 4. Basic patterns of a thumb print.

Each friction ridges taken together with other features including crossover, core, bifurcations, ridge ending, island, delta, and pore, make up the fingerprint pattern (Fig. 5)

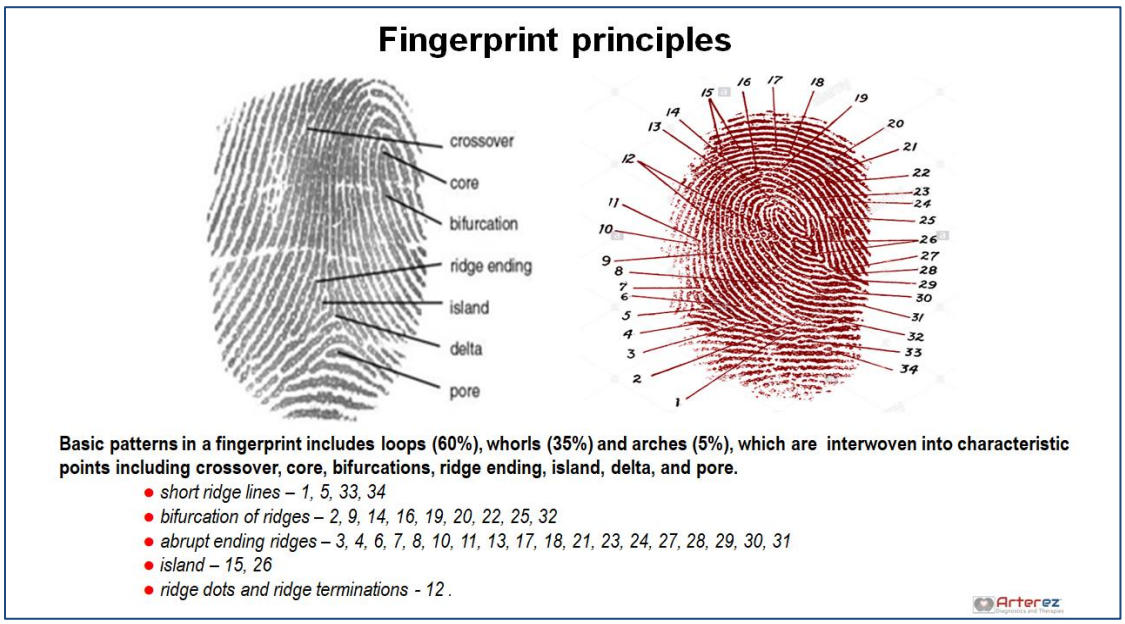

Figure 5. Each fingertip print is unique to an individual, which is the basis of fingerprint.

2. **Genomic parameter** - 1984: Sir Alec Jeffreys (UK) invented the DNA fingerprinting (genetic profiling) technique. Unlike the physical patterns from fingertips, a DNA fingerprint is based on genetic patterns involving nucleotide sequences. Certain parts of the DNA, which is about 0.1% or 3 x 10^6 base pairs (out of 3 x 10^9) possesses numerous small noncoding but inheritable sequences of bases. Depending upon length, these base sequences are termed satellite DNAs with subcategories like mini-satellites and microsatellites, which are very specific in each individual. In particular, the mini-satellites are characterized with 'Variable Number Tandem Repeats' (VNTRs), which are used as the genetic markers to identify individuals in paternity/maternity disputes, human lineage, hereditary diseases, forensics and ethnic origin:

   Briefly, the DNA molecules are recovered with the help of enzyme restriction endonuclease (called chemical knife) that cuts them into fragments. The minisatellite fragments are separated from the bulk DNA during density gradient centrifugation then further resolved according to size by gel electrophoresis. Fragments of a particular size having VNTRs are transferred onto a nylon membrane. Radioactive DNA probes having repeated base sequences complementary to VNTRs are poured over the nylon membrane, which bind or hybridize to the VNTRs (called Southern Blotting, after the name of the inventor, E.M. Southern, 1975). An X-ray film is exposed to the nylon membrane to mark the radioactive dark bands, which represent the DNA profile or fingerprint (Fig 6).

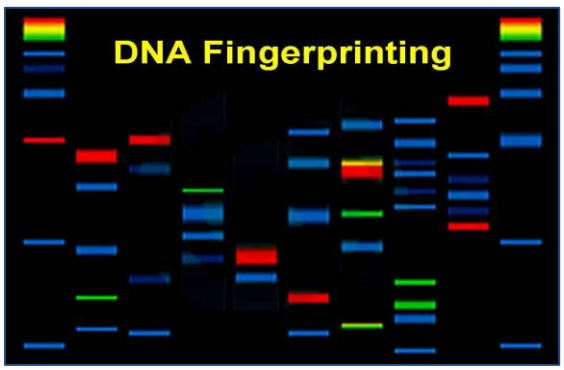

Figure 6. DNA bands observed in an electrophoretic gel is a unique feature of an individual

To compare two or more different DNA fingerprints, DNA samples are run side-by-side on the same electrophoresis gel (Fig. 7)

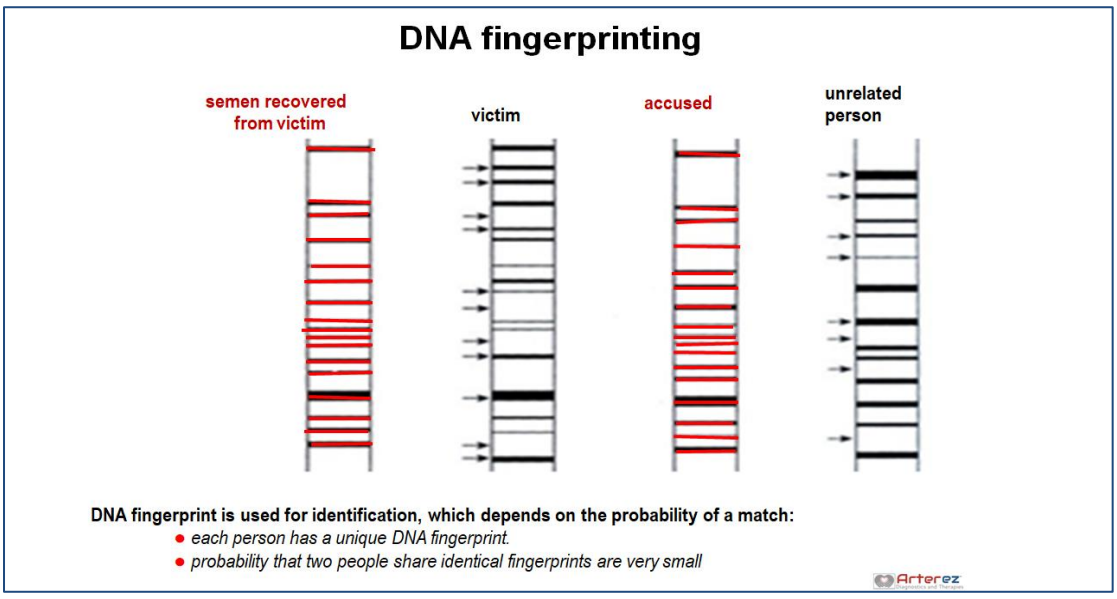

Figure 7. Matching DNA fingerprints of sample recovered in a crime site and the accused establishes connection.

3. **Detritus parameter** - Dr. J. B. Tunac (US) first introduced the hypothesis of glycocalyx detritus *(rubbed or worn off glycocalyx debris)* as component blood biomarkers for a biological fingerprint in 2012. Currently, there is no equivalent fingerprint system developed for disease diagnosis. In this regard, the glycocalyx detritus pattern presents an equivalent to the physical patterns found on fingertips as a basis for the classic fingerprint orD the nucleotide microsatellites bands that describe a DNA fingerprint. The classic fingerprint and DNA fingerprint do not diagnose diseases and are only used to identify individual humans from another. On the other hand, a panel of markers, clinically correlated to one or more root causes of vascular diseases, can be designed to identify, predict and diagnose those diseases, an area that could revolutionize or mark a new era in healthcare. As opposed to a single biomarker, this not only increases the accuracy of diagnosis but also allows disease identification, classification and disease staging serving as guide for improved therapies. Arterez is already in the process of developing panels, or fingerprint for CVD diseases, we refer to as Glycocardia™. For instance the CHD panel is Glycocardia^{CHD} while the heart failure panel is Glycocardia^{HF} etc.

   The glycocalyx contains anchoring proteoglycans (such as CD44) and members of the syndecan protein family, as well as connecting glycosoaminoglycans, such as heparan sulfate, chondroitin sulfate and hyaluronic acid. Under conditions of oxidation and inflammation, the glycocalyx begins to break down, releasing detritus components, leading to endothelial damage and a cascade of pathological conditions. For example, the release of microparticles (storage pool of bioactive cytoplasmic proteins and lipids involved in a variety of fundamental processes), detachment of the pericytes and circulating endothelial cells (CEC); increase in CECs precede that of established tissue-damage markers like troponins or creatine kinase. CECs counts are very low in healthy individual but elevated in patients with diabetic nephropathy (DN), heart failure with preserved ejection fraction (HFpEF), heart failure with reduced ejection fraction (HFrEF), and arterial hypertension (aHT).

Glycalyx Detritus Fingerprint™ Technology (GDF) – Proof of Principle

The rapid and correct identification of diseases is crucial and important as a guide for appropriate therapy. 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 and symptoms are nonspecific. For example, redness of the skin, allergy, cardiovascular disease, diabetes, or cancer are each a sign of many disorders and differential diagnosis must be performed to improve accuracy. This involves the correlation of various pieces of information followed by the recognition and differentiation of patterns or ‘fingerprints’.

The GDF is a first in kind technology that could revolutionize or mark a new era in healthcare. This "biomarker panel" technology, as opposed to a single biomarker, increases the accuracy of diagnosis and enables disease identification, classification and disease staging serving as guide for improved therapies that target the multi-factorial root causes of chronic disease, specifically damage to the GCX. Proof-of-principle research/studies include:

a) 4-Panel GDF (Glycocardia^{CHD}) as a companion diagnostic for plaque formation

Coronary heart disease (CHD) is a member of the cardiovascular family (CVD) and leading disease killer in the world. CHD characteristic feature is plaque formation, which results in atherosclerosis or hardening of the arteries. Plaque formation is triggered by glycocalyx disruption and the shedding of glycocalyx detritus. In this regard 4 glycocalyx detritus (*Glycocardia^{CHD}*) were selected as components of the fingerprint, namely: syndecan-1 (SDC-1), heparan SO_4 (HS), hyaluroman-1 (HAS-1 :), and plasminogen activator inhibitor -1 (SDC-1). Dr. Tunac also developed the Tunac Arterial Plaque (TAP) Natural Mouse™ model, used to model plaque formation as it occurs in humans. Indeed, the blood levels of the 4 detritus correlated with plaque formation (Fig 8):

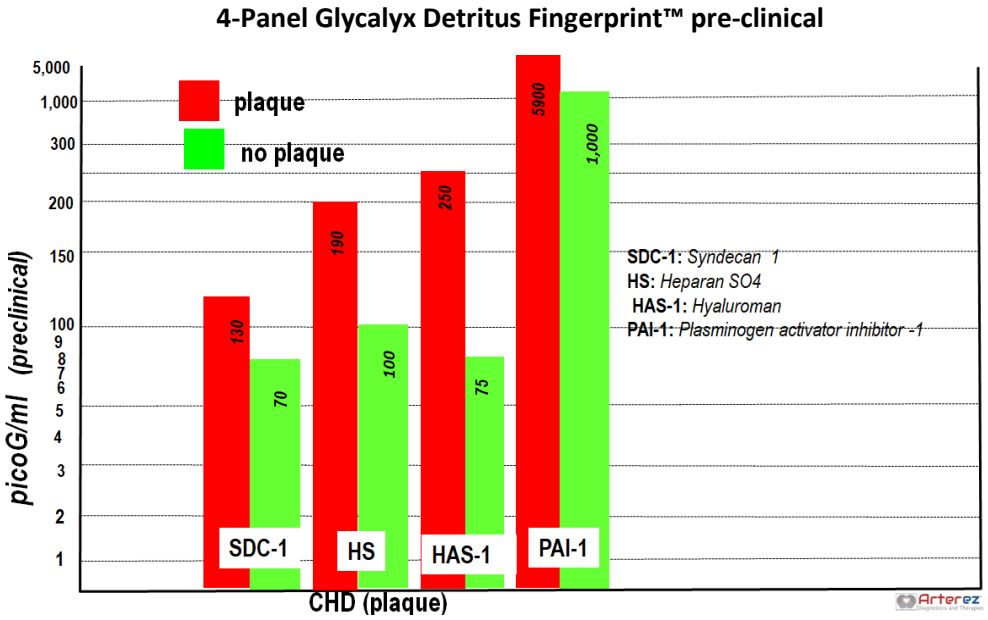

Figure 8. Fingerprint of a 4-panel glycocalyx detritus showing elevated blood levels in the presence of plaque and a corresponding decrease without plaque.

b) 5-panel GDF (*Glycocardia^{HF}*) – Clinical Study

The correlation of blood levels of the 4 glycocalyx detritus to plaque formation prompted the evaluation of IRB clinical samples. These clinical samples represented blood drawn from patients suffering from chest pain, heart failure (HF) and hypertension (HTN);

Cardiac troponin (cTn), proteins found in skeletal and heart muscle fibers is the most common diagnostic tool in the ER. The test is ordered if a person is experiencing symptoms such as: chest pain (angina), shortness of breath (heart failure), and hypertension (rapid heart rate, lightheadedness, fatigue). Even with the widespread use of cTn assays worldwide, there remains some confusion among clinicians and laboratorians about the timing, frequency, and duration for measuring cTn after patients present with symptoms suggestive of acute coronary syndrome (ACS) highlighting the discrepancies and errors that can occur when relying on a single biomarker. For this reason, the 5 glycocalyx detritus were selected, namely: growth differentiation factor-15 (GDF), plasminogen activator inhibitor -1 (PAI-1), pregnancy associated plasma protein –A (PAPP-A), syndecan-1 (SDC-1), and heparan SO_4 (HS). Blood levels of these 5 detritus were evaluated using ELISA. The levels of the 5 glycocalyx detritus were elevated in each of the diseases; moreover, each disease produced a characteristic pattern or fingerprint (Fig. 9).

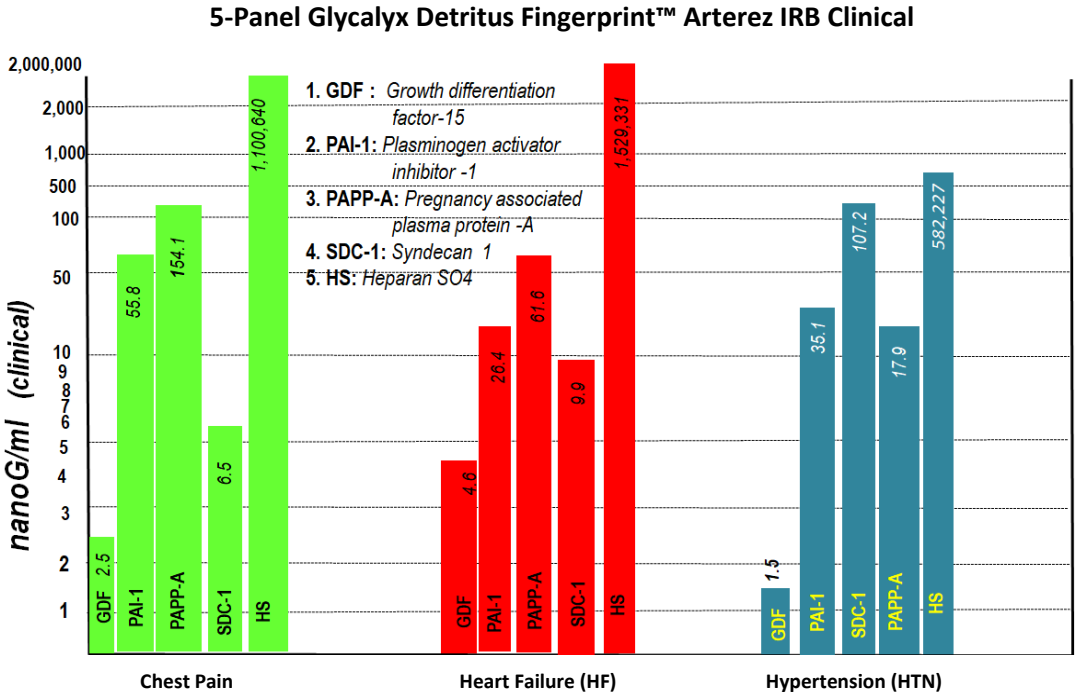

Figure 9. Fingerprint of 3 diseases (chest pain, heart failure, hypertension) which are members of the CVD family, showed significantly different levels of each of the biomarkers, differentiating each disease from the other.

c) Direct and indirect glycocalyx detritus as components of the GDF.

Glycocalyx detritus directly or indirectly start the disease cascade, including the triggering or generation of other detritus into the bloodstream. Such indirect detritus include growth differentiation factor -15 (GDF-15), pregnancy associated plasma protein–A (PAPP-A) and gamma fibrinogen (GF). Direct and indirect glycocalyx detritus can be incorporated as components of a disease fingerprint system (Fig 10).

GlycoCardia™ panel and diagnostic disease targets

- Heparan SO4 (HS)– **ischemia , hemorrhagic, hypertension** *(2007. Circulation116:1896-1906; 2002. Acta Obstet Gynec Scan 81:308; 2017. Scientific Reports 7, No 46191)*
- Plasminogen activator inhibitor -1 (PAI-1) – **stroke** *(2005. JClinNeurol2:142-147)*; CHD *(214. Addict Health 6:119-126)*; acute MI *(2014. Int J Clin Exp Med 7:1059-1063)*
- Syndecan 1 (SDC-1)– **heart failure, renal failure ischemia, acute coronary syndrome** *(2015. Circulation79:1511-1519; 2016. Atherosclerosis 247:184-188; 2007. Circulation 116:1896-1906; 2012. JASN 23:1900-1908; 2015. Br J Clin Pharmacol 80: 389–402)*
- Hyaluronan (HA)– **stroke** *(2014. JNeuroinflammation 11:101); hypertension (2013. TohukoJExpMed 230:7-11)*
- Gamma (γ') fibrinogen (GF) – **myocardial infarction and stroke** *(2007.J Thromb Haemost 5:766 – 73; 2012. Thrombosis Res 129: 807-809*
- Growth differentiation factor 15 (GDF-15) – **heart failure, coronary artery diseases, atrial fibrillation, diabetes** *(2013. Clinical Chemistry 59: 1550–1552; 2014. Circulation 130:1847–1858; 2012. Clinical Chemistry 58: 172–182.*
- Pregnancy associated plasma protein (PAPP-A) – **rupture-prone plaque** *(2016. Medicine (Baltimore). 95(3): e2563;2012. Cardiovasc J Afr 23:330–335;2015. Biomark Med 9:731–741; 2016. Medicine (Baltimore) 95:e2563; 2004. Circulation 109:1724–1728;2005. Clin Chem 52:1096–1103.*

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Figure 10. Example of direct and indirect glycocalyx detritus as part of the GDF

d) 7-Panel GDF (Glycocardia^{GEN}) – Clinical Data

Blood levels of 7 detritus components were obtained from published literature of patients with coronary heart disease (CHD), heart failure (HF), and hypertension and a virtual fingerprint was constructed. Each disease showed a unique fingerprint, which confirms the effectiveness of the GDF as a unique tool for identifying diseases (Fig 11).

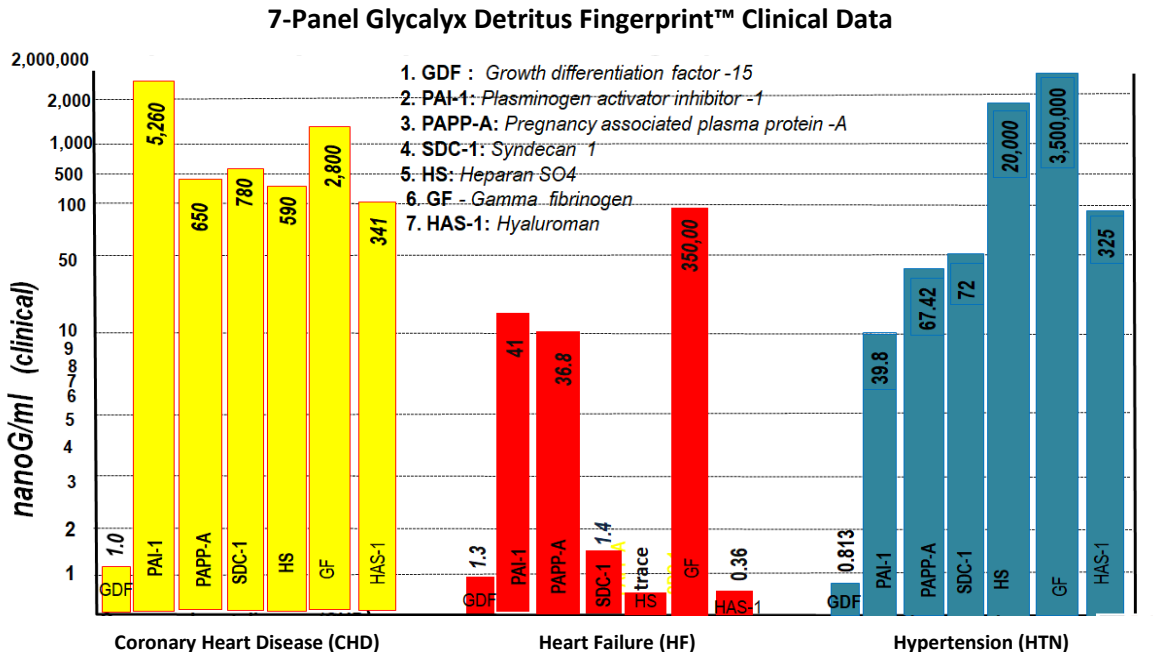

Figure 11. 7-panel detritus showing unique profile for 3 diseases: coronary heart disease (CHD), heart failure (HF), and hypertension (HTN)

Glycalyx Detritus Fingerprint™ (GDF) – Opportunity & Potential

The GDF proved to be effective in creating distinct patterns or fingerprints of the different family members of CVD tested. While the classic fingerprint and DNA-fingerprint techniques were primarily designed to identify individuals in the field of forensics, the GDF is the first analytical tool to diagnose diseases, a new era in healthcare to assist in targeted treatment. As stated earlier, we believe this technology will increase the accuracy of diagnosis and enable disease identification, classification and disease staging serving as guide for improved therapies targeting endothelial repair, beginning with Arterez 3x combo therapy, Embotricin™ that specifically targets glycocalyx repair, inflammation and minimizing oxidation.

Current developmental efforts and objectives include:

- Establish and test the 7-panel Glycocardia panel as a companion diagnostic tool for our 3x combo therapy to be tested in parallel, in clinical trials by Q3 2021.
- Combine the 7-marker (Glycocardia) panel into our issued patent for same (completed October, 2019).
- Build a robust database for chronic disease, initially heart failure, hypertension and coronary heart disease, while piloting other vascular pathophysiologies such as Alzeimer's, Arthritis and Type 2 Diabetes (Fig 12).
- Integrate a statistical analysis process to begin to evaluate clinical data toward 3-5 marker panel "fingerprints" for individual diseases resulting in proprietary algorithms derived from our centralized disease repository.
- Use a combination of antibodies and mass spec in a single platform, enabling a single molecule analysis, 1,000x more sensitive than conventional ELISA, as the standard operating procedure (SOP) to standardize or centralize all detritus blood levels and eliminate disparities among laboratories.
- Identify and engage a co-development partner, enabling and accelerating the development process toward commercialization of individual diagnostic panels in parallel to clinical studies as outlined above.
- Consider the integration of available and emerging technologies, including biochip and artificial intelligence as appropriate in context of the development process and commercialization strategy TBD.

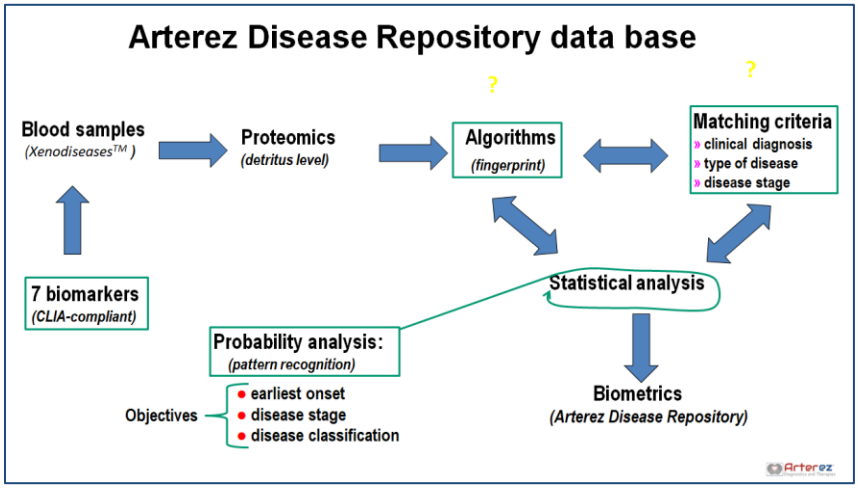

Figure 12. Development pathway and establishment of an Arterez Disease Diagnostic Repository data base

Statistical Analysis

We've engaged an analyst to begin to evaluate our findings as we build the repository. Following in an overview of the proposed statistical process and methodology which will be further honed and implemented in order to discern and create Detritus Fingerprints and resulting algorithms. This will be accomplished by using the 7-panel matrix (Glycocardia^{GEN}) in order to arrive at a path analysis to establish causality between and amongst the biomarkers and cardiovascular disease. As the data set expands and the studied patient population expands (as contained within the Arterez Disease Repository database), this statistical model will be refined to accommodate.

The statistical study will be done in several steps to underpin the data as a system of checks and balances for each data set. Several of these steps will provide rapid access to demographic and other data within the patient population. Later phases of the study will provide confirmation of predictability of the 7-panel matrix.

Phased Analysis Approach

1. The first phase of the study will be a frequency distribution of the patient demographics as well as the proteins. This will confirm counts, help identify faulty coding (for individual variables), and rapid access to the case counts and their relative frequency in the study.
2. The second cursory analysis of the data will involve the cross tabulation of the disease processes we study within the CVD family to include hypertension (HTN), heart failure (HF) and coronary heart disease (CHD). This will give some idea of trend analysis of the data as well as an indicator of what more robust data analysis should provide. It will also provide validation of the coding mechanism that may not have arisen in the first step as we are looking at several variables at a time rather than individually.
3. A correlation matrix will be created to measure the strength, direction, and in some cases, the significance of the variables through their interplay with one another. This simply permits us to delve further into trend analysis, as in the second facet, but not predictability.
4. After correlations are completed, a multiple regression will be conducted. This technique measures the level of predictability that we can achieve when looking at the independent variables and how they act upon the dependent variables. In our study, the independent variables will be the 7-panel biomarkers and the dependent variables (initially) will be HTN, HF, and CHD.
    a. This type of F test will permit us to set the level of significance we would like to see and ignore those relationships that fall below that level. It will provide a "beta" value which is somewhat equivalent to the "slope" of a line indicating the positive/negative relationship between two variables. That is to say, if the relationship is positive, the two variables increase or decrease together.

If it is negative, then one or the other increases/decreases while the other moves in an opposite direction. A multiple regression will also report the R^2 value. The R^2 value roughly translates into the percentage of the variance that we can explain with the variables. Multiple regression will further permit us to review the standard error among the variables to further increase our confidence in the relationship of the data.

5. Path analysis uses regression analysis to illustrate the relationships between the variables. It will also show relationships between the independent variables should the "path" to the dependent variable not only have a relationship with one, but with multiple variables "through" one another. This model will permit us to see the various relationships and where. While seemingly complicated, when the statistical data is applied, it will show the relationships all seven (7) variables have on each dependent variable individually, and also show how the variables interact with each other, prior to looking at their relationship with the dependent variables. The complexity of the study will permit us to understand A →B relationships as well as A→B→C Relationships and a more complete understanding of the model.
6. As a final sixth step, we will confirm the results of the path analysis by conducting a MANOVA test. The MANOVA test will use the covariance of the variables to test their significance to each of the dependent variables separately. This process will essentially check the veracity of the path analysis for its predictive ability.

Our intent is to move past the benchmark of proving the "fingerprint" model, and into the realm of predictability. With that in mind, the data will drive the ability to create a suite of tests that will be administered and conducted to more correctly identify and perhaps predict and or diagnose individual diseases. As the data set grows, the data should also be useful in fingerprinting the various stages of each disease within each individual patient, resulting in more pointed, appropriate and pertinent treatment options ultimately reducing morbidity and mortality.

Glycalyx Detritus Fingeprint™ - multiple uses

The Glycalyx Detritus Fingerprint™ can be used twofold: 1) a companion diagnostic for custom therapies (e.g., Embotricin™), or 2) 'stand-alone' diagnostic to monitor or evaluate traditional symptom-targeted therapies (Fig 13).

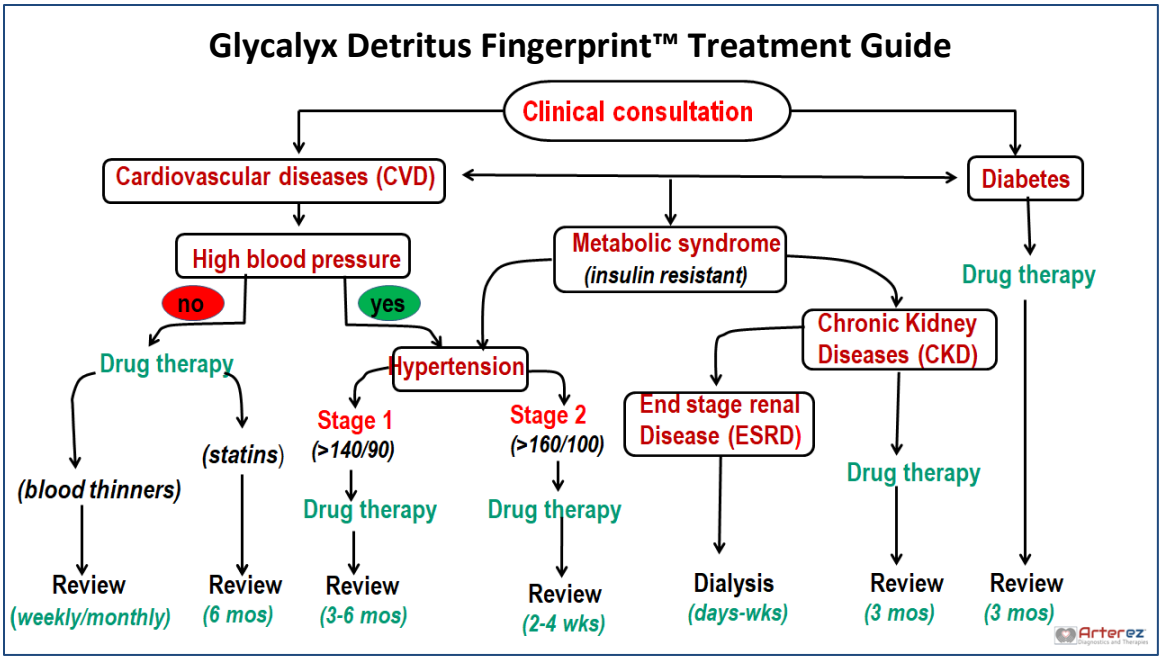

Figure 13. Scheduled use of the Glycalyx Detritus fingerprint™ to monitor Embotricin™ or traditional symptom-targeted therapies.