Accurate diagnosis for diseases: The "Glycalyx Detritus Fingerprint™"

Dr. J. B. Tunac (1/15/21)

Highlights:

- *Arterez has developed a diagnostic tool for chronic diseases based on a "fingerprint' platform, herein called the* ***"Glycalyx Deritus Fingerprint™"*** *(GDF) .*
- *Fingerprinting, which involves a multicomponent set of parameters, is the most accurate system for identification or diagnosis.*
- *While the classic "thumb print" (based on the unique physical contour of fingertips) and the "DNA fingerprint" (based on unique DNA pattern) accurately identify individuals in forensics, paternity, or ethnic origin, GDF is the first fingerprint to diagnose diseases based on unique shedding patterns of the vasculature lining called the glycocalyx.*
- *GDF marks a new era in healthcare to assist in early and accurate disease diagnosis for targeted treatments.*

A. Glycocalyx (GCX) disruption as the root cause of multiple chronic diseases.

1. Glycocalyx is the first line of defense to proper blood flow

Body cells are organized in 4 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 tissues: protect underlying tissues from radiation, desiccation, toxins, pathogens, and physical trauma; regulate exchange of chemicals between tissues and a body cavity; secrete hormones into the blood vascular system, provide 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 glycocalyx, which provides the first line of protection from physical, chemical, and biological wear and tear (Fig. 1)

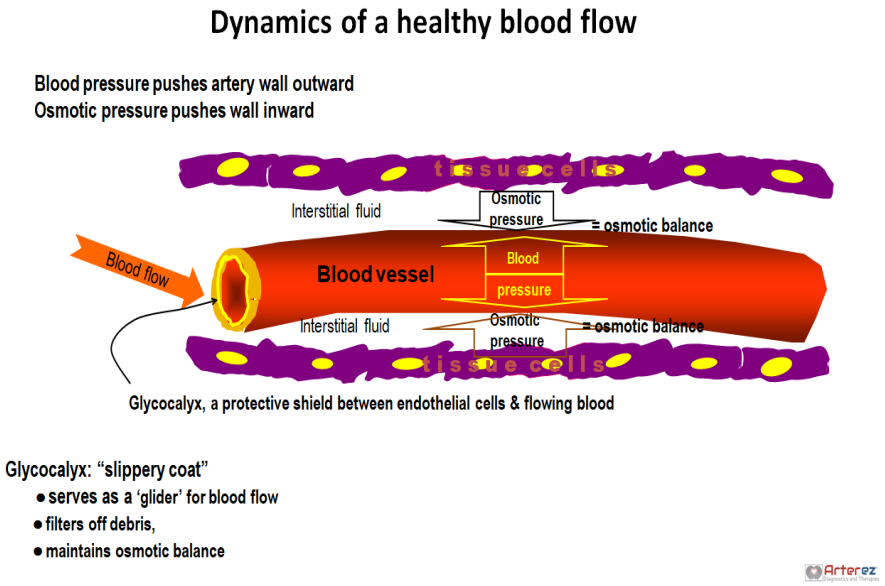

Figure 1. Blood vessel is lined with a 'slippery coat' called glycocalyx that promote healthy blood flow dynamics.

2. Glycocalyx consists of multicomponent parts, interwoven to maintain vessel integrity

Glycocalyx 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 glycocalyx include: 1) transmits fluid shearing forces to the cytoskeleton of endothelial cells and stimulates the production of nitric oxide, which is vital in controlling blood flow and blood pressure; 2) regulate the supply of nutrients and oxygen, and the removal of waste and carbon dioxide; and 3) maintains capillary integrity, and prevents loss of fluid through leakage (Fig. 2)

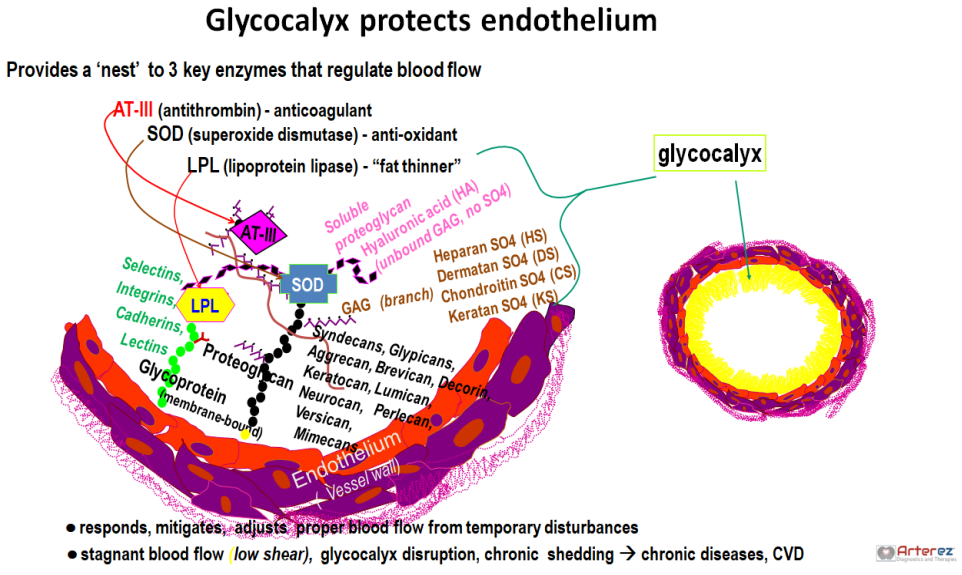

Figure 2. Anatomy of the protective endothelial glycocalyx showing glycoprotein and proteoglycan components including nested proteins (AT-III, SOD, and LPL)

Glycocalyx 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].

3. Disruption of glycocalyx 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)

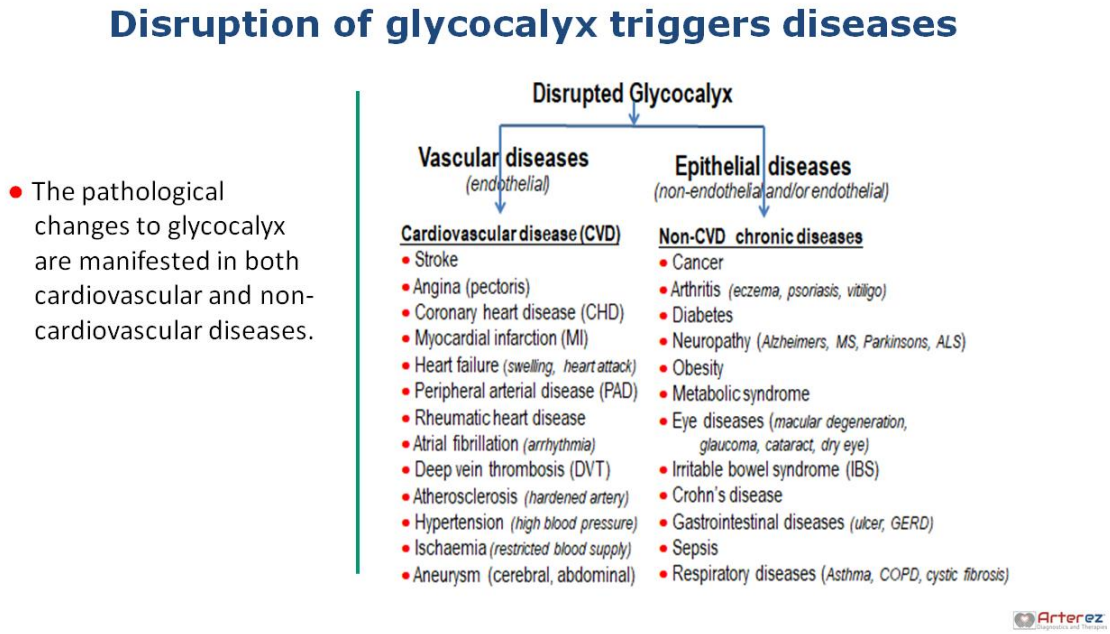

Figure 3. Disruption of the glycocalyx accounts for several vascular-related pathophysiologies including CVD

4. Recent reviews supporting glycocalyx disruption as the root cause of several pathologies, basis for diagnostics and therapeutic target:

https://journals.lww.com/cmj/Fulltext/2019/04200/Endothelial_glycocalyx_as_a_potential_theriapeutic.11.aspx
Chinese Medical Journal: April 20, 2019 - Volume 132 - Issue 8 - p 963–975
Endothelial glycocalyx as a potential therapeutic target in organ injuries
Cao, Rui-Na; Tang, Li; Xia, Zhong-Yuan; Xia, Rui

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6337861/
Crit Care. 2019 Jan 17; 23(1):16.
The glycocalyx: a novel diagnostic and therapeutic target in sepsis.
Uchimido R, Schmidt EP, Shapiro NI.

https://jintensivecare.biomedcentral.com/articles/10.1186/s40560-016-0182-z
Journal of Intensive Care volume 4, Article number: 59 (2016)
Glycocalyx and its involvement in clinical pathophysiologies
Akira Ushiyama, Hanae Kataoka & Takehiko Iijima

https://academic.oup.com/cardiovascres/article/87/2/300/446026 -
Cardiovascular Research, Volume 87, Issue 2, 15 July 2010, Pages 300–310
Therapeutic strategies targeting the endothelial glycocalyx: acute deficits, but great potential†
Bernhard F. Becker, Daniel Chappell, Dirk Bruegger, Thorsten Annecke, Matthias Jacob

B. Glycocalyx debris (detritus) as components of a fingerprinting system

1. Fingerprints as identification and diagnostic tools

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, symptoms are nonspecific. For example, redness of the skin, allergy, cardiovascular disease, diabetes, or cancer each taken by itself, is 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 idea of fingerprinting started in the primitive times when man used to hunt for food with the help of animal’s footprints. Since then, the science of ‘fingerprinting’ evolved as an identification system using a set of parameters:

a) **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 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)

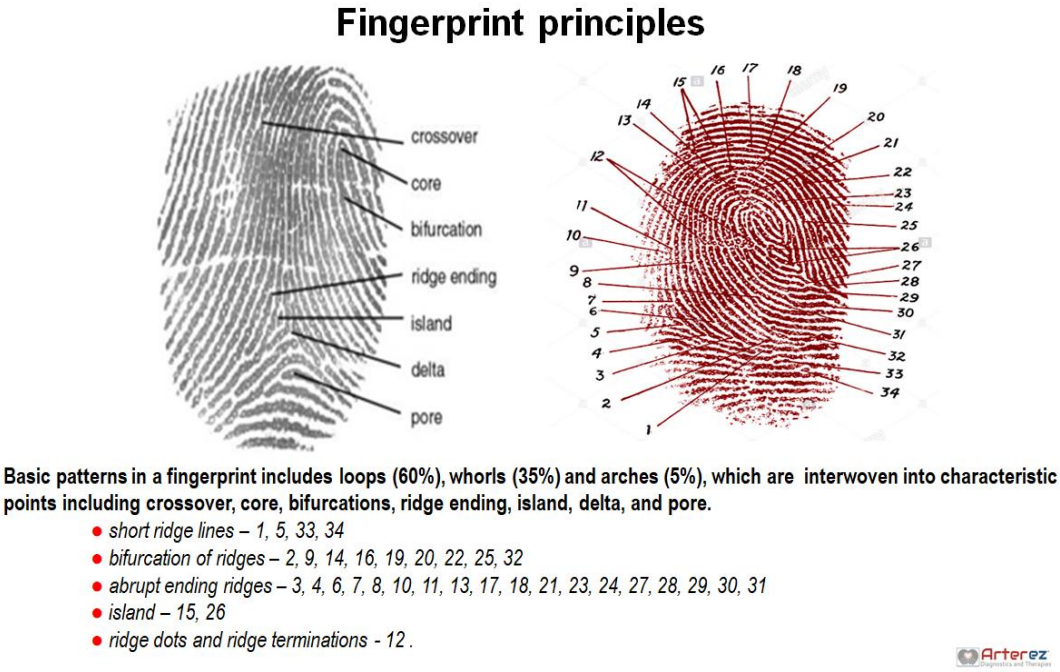

Figure 5. Each fingertip print is unique to an individual, which is the basis of fingerprint.

b) **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 extremely 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).

To compare two or more different DNA fingerprints, DNA samples are run side-by-side on the same electrophoresis gel (Fig. 6)

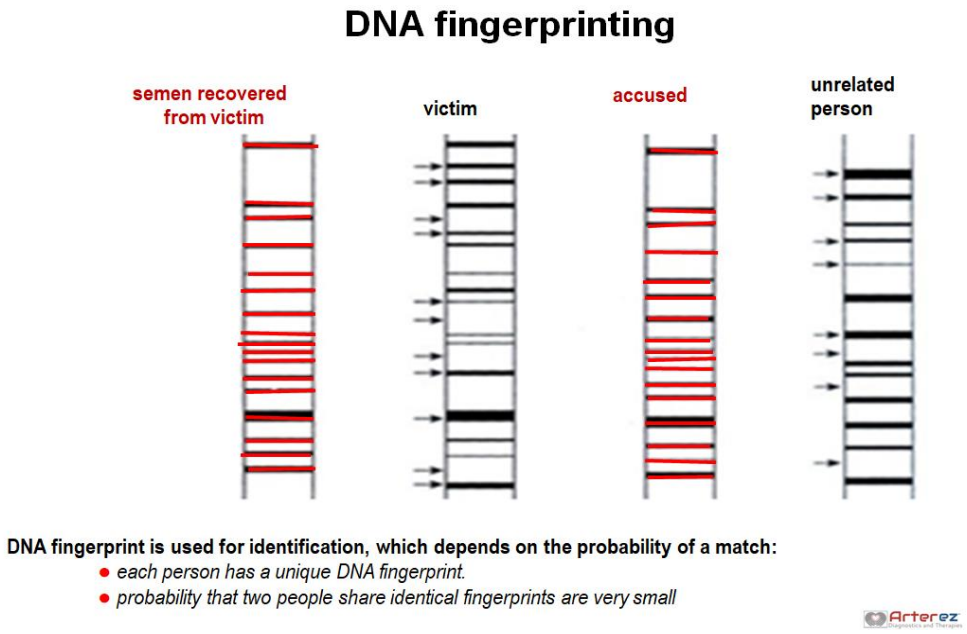

Figure 6. Matching DNA fingerprints of sample recovered in a crime site and the accused establishes connection.

c) **Detritus parameter** - 2012: Dr. J. B. Tunac (US) introduced glycocalyx detritus *(worn off glycocalyx fragments)* as components for a biological fingerprint. Currently, there is no fingerprint system developed for diseases. Thus, the glycocalyx detritus shedding pattern becomes the equivalent to the physical patterns found on fingertips or the nucleotide microsatellite pattern found in DNA bands. The classic fingertip pattern and DNA microsatellite pattern do not diagnose diseases but are used to identify individual humans in forensics or paternity cases. On the other hand, glycocalyx detritus pattern is the first in

kind to diagnose diseases a new dawn in healthcare. A fingerprinting system for diseases not only increases the accuracy of disease diagnosis but allows for disease identification, classification and staging serving as guide for improved therapies.

Briefly, glycocalyx is a slimy protective coat that lines the endothelium, which 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 extremely 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).

2. Proof-of-principle: utility of glycocalyx detritus as basis for a fingerprint

a) Preclinical proof: 4-panel glycocalyx detritus as a fingerprint for plaque formation *(Glycocardia™)*

Coronary heart disease (CHD) is a member of the cardiovascular family (CVD) and the leading CVD killer. The characteristic feature of CHD 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 a separate project, Arterez developed a preventive and curative drug treatment (Embotricin) for plaque in an animal model (preclinical stage). The paradigm for this project is that disruption of the glycocalyx exposes the cell membrane to injury creating tiny holes for entry of blood debris and plaque formation (Fig. 7).

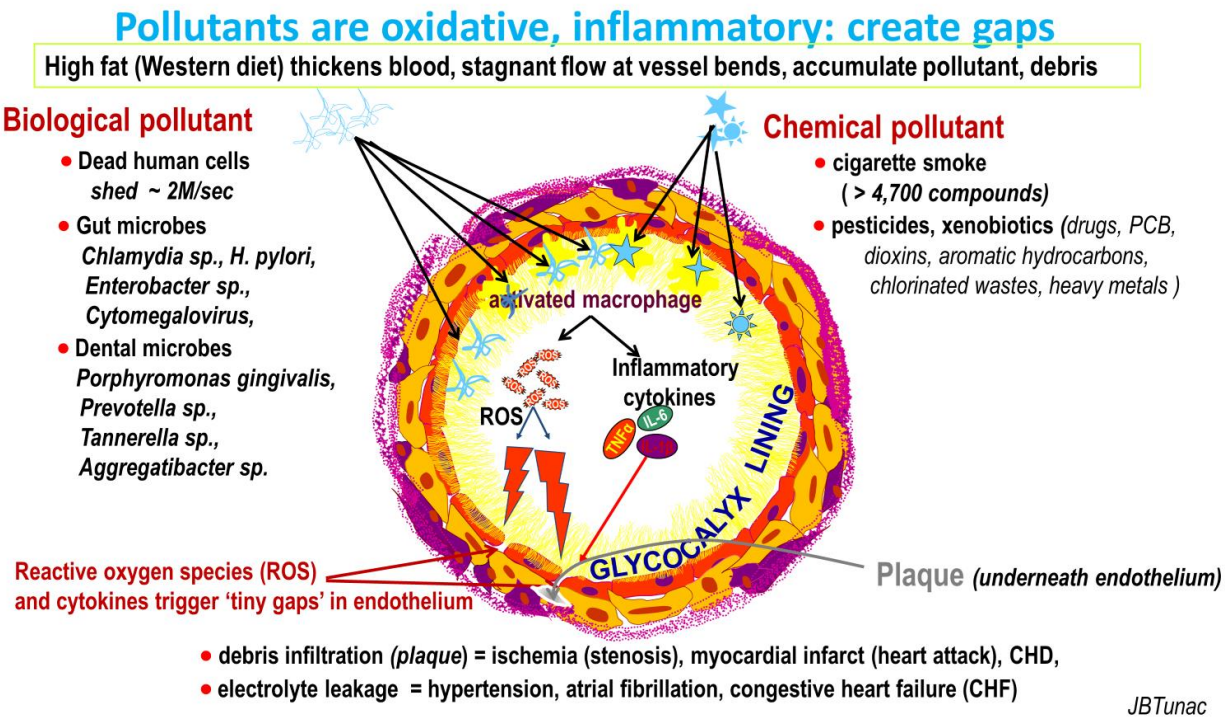

Figure 7. Cross section of an artery showing plaque formation due to the oxidative and inflammatory effects of xenobiotics

Indeed, plaque formation is central to CVD and sans histopathology, a diagnostic system is needed to follow plaque formation. Plaque formation is a complex process and there is no single biomarker known to monitor this event. Thus, a panel of biomarkers was conceived, particularly a 4-panel glycocalyx detritus including syndecan-1 (SDC-1), heparan SO4 (HS), hyaluroman-1 (HAS-1), and plasminogen activator inhibitor -1 (SDC-1), herein called **(*Glycocardia*™).**

The utility of ***Glycocardia*™** was highlighted during the development of **Embotricin™**. Briefly, a natural mouse (Jackson Labs) was xenobiotically induced (exposure to environmental pollutants and high fat diet) to produce plaques. These are factors that mimic the environmental and lifestyle risk factors in humans. For the project, plaque-bearing mice were created by feeding high fat diet and given PCB pollutant ***(The Tunac Arterial Plaque model).*** Twelve drug compounds (A-L) were evaluated for preventive (before plaques were formed) and curative (after plaques were formed) using the 4-panel glycocalyx detritus to monitor drug effect. Untreated mice (no drug compound) were set aside as control, which showed elevated ELISA blood levels in each of the detritus markers and reduced level corresponding to efficacy of the drug compound. The net drug effect is the difference between the ELISA detritus level of untreated and drug treated, where a 'positive bar' indicates no detritus shedding (no glycocalyx disruption), and 'negative bar' means detritus shedding (disrupted glycocalyx) (Fig. 8).

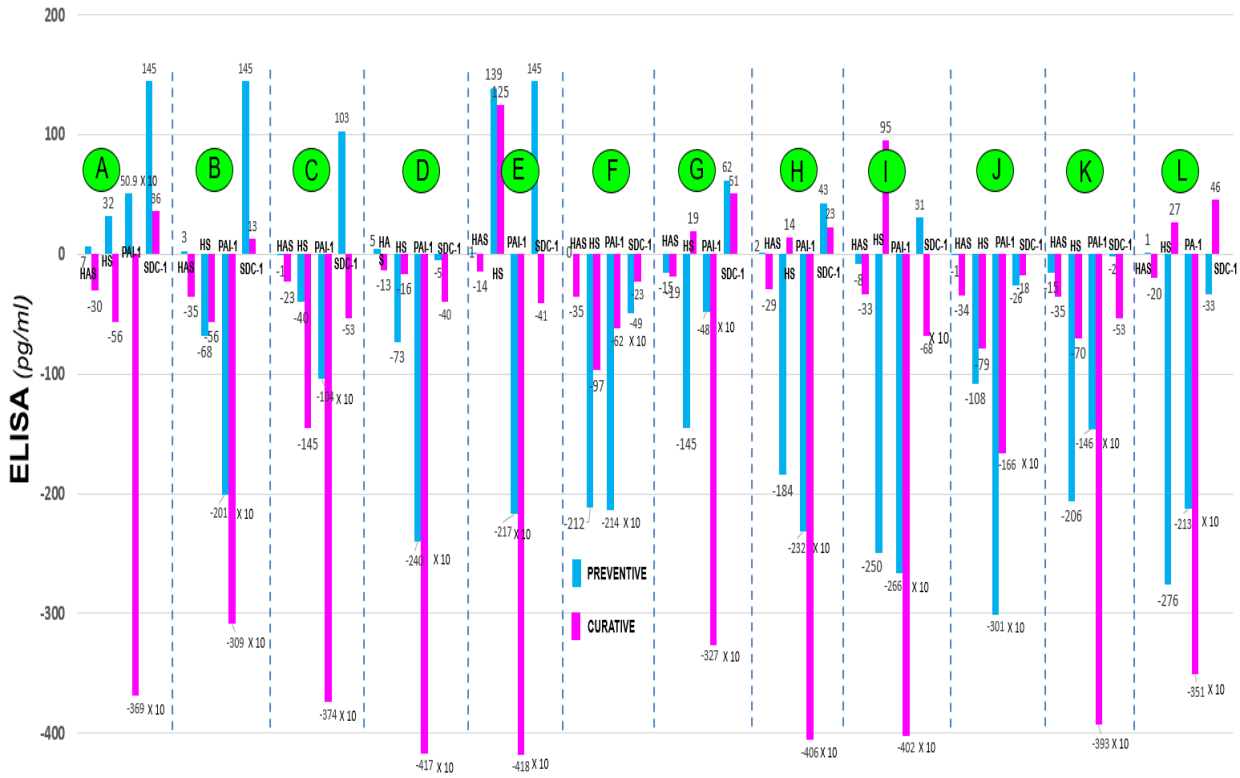

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Figure 8. Activity pattern of the 12 compounds (A-L) per 4-panel Glycocardia where a 'positive bar' indicates no glycocalyx disruption and 'negative bar' means disrupted glycocalyx.

For example, compound A shows 'positive bars' in the preventive column and 'negative bars' in the curative column (except SDC-1) indicating combo A as a curative but not preventive treatment. Based on the ***Glycocardia*™** profile, 4 compounds showed curative/preventive effects: K >J > F > D. Compound K was ranked the highest because of its overall superior profile in all the markers and hereby designated **Embotricin™**. More importantly, the 4-panel ***Glycocardia*™** correlated with histopathology (Fig. 9)

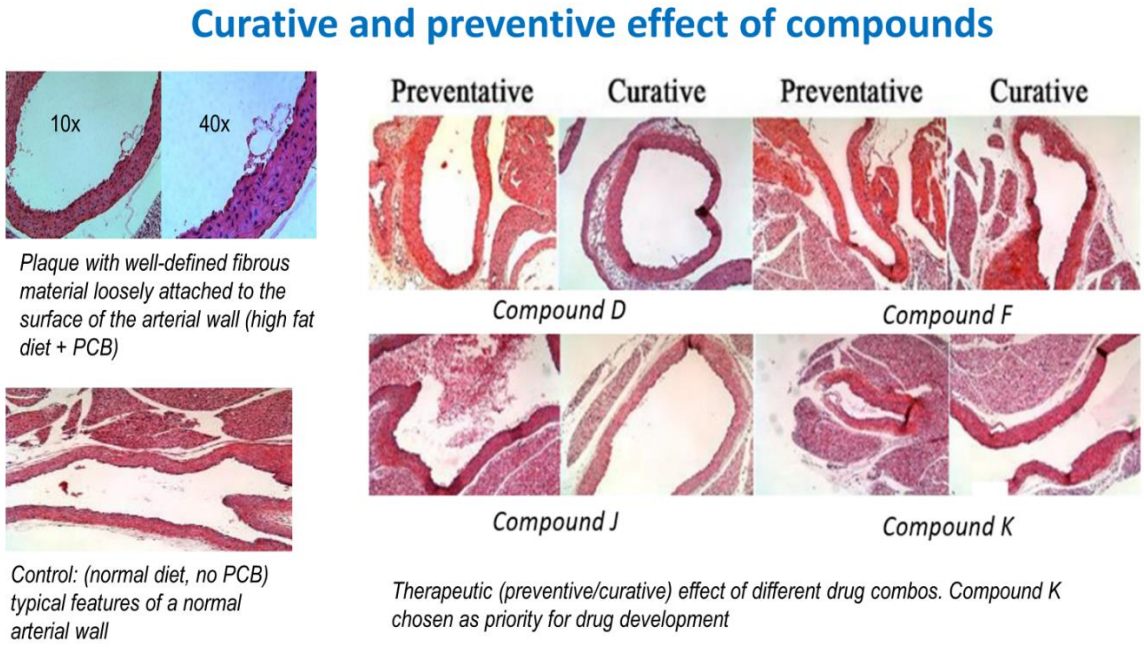

Figure 9. Histopathology of brachiocephalic arteries of mice treated with compounds D, F, J and K showing no plaques, while untreated artery (control) showed plaques.

b) Clinical proof-of-principle

The above preclinical trial established the correlation between a 4-panel detritus and histopathology regarding monitoring plaque formation. Plaque formation is central to CVD, particularly the family of coronary heart disease (CHD). The question remains whether the preclinical observation is translatable to clinical samples and whether the concept of a panel is applicablein diagnosing other diseases in the CVD family.

Cardiac troponin (cTn), proteins found in skeletal and heart muscle fibers, is the most common diagnostic tool in emergency room (ER). The test is ordered if a person is experiencing possible 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 discrepancy of a single biomarker. For this reason, 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 by ELISA.

The levels of the 5 glycocalyx detritus were elevated in each of the diseases. More importantly, each disease produced a characteristic pattern different from each other, which indicates the

potential of establishing a "fingerprint" system for chronic diseases as a counterpart to the classic fingertip and DNA fingerprint system (Fig. 10)

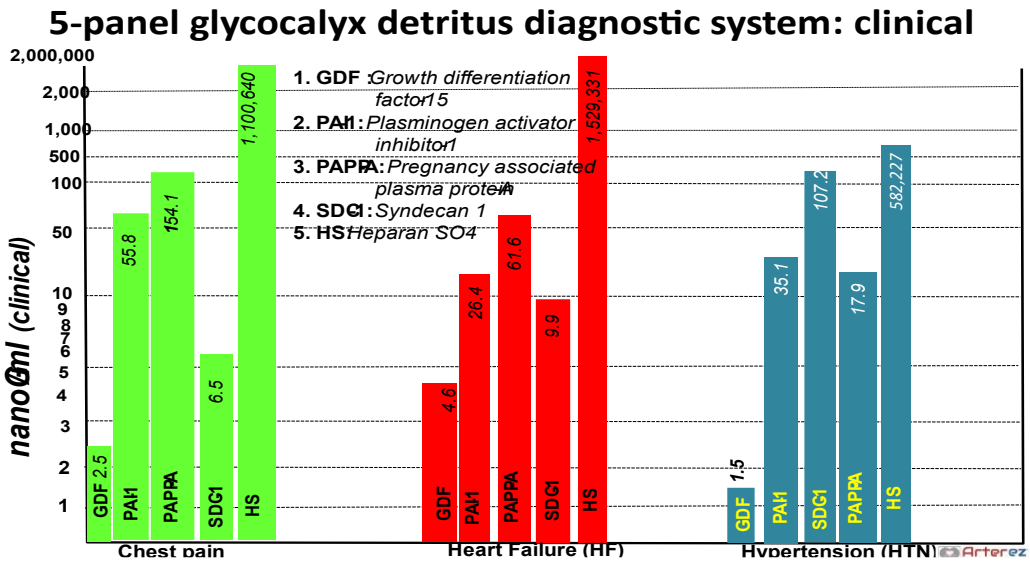

Figure 10. ELISA profile of 3 diseases (chest pain, heart failure, hypertension) showing patterns or fingerprint unique from each other.

c) The Glycalyx Detritus Fingerprint™ vs clinical samples

The above 5-panel proved the utility of a fingerprinting system for diseases in the CVD family for clinical use. To expand the concept of "fingerprinting" as a diagnostic platform across chronic diseases (see Fig 3), Arterez developed the "Glycalyx Detritus Fingerprint™", a 7-membered biomarker panel. (Fig 11).

The 7-panel Glycalyx Detritus Fingerprint™

- 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 11. Example of direct and indirect glycocalyx detritus as part of the glycocalyx detritus fingerprint™

d) Experimental proof of principle:

Serum blood (IRB) samples were obtained from Trans-hit Bio (Laval, Quebec Canada) drawn from patients clinically identified with chronic diseases, including coronary heart disease (CHD), heart failure (HF), rheumatoid arthritis (RA), stroke, hypertension (HTN), diabetes 2 (DIAB), Alzheimers (ALZ) and healthy (HEALTH) individuals. These blood samples were analyzed per ELISA vs the GDF for levels of detritus biomarkers (courtesy of Arbor Assays Arbor Assays, Inc. Ann Arbor, MI)

To establish a standard operating procedure (SOP) for the GDF, both serum and plasma were evaluated. The results show that ELISA levels of the markers were generally comparable in both serum and plasma samples (Fig 12). However, no evaluable levels were found for gamma fibrinogen in the serum samples as illustrated in the bar chart (Fig. 13). In this regard, plasma preparation is be the preferred sample moving forward to build an "Arterez Disease Repository".

Thus, the observed sequence of blood marker levels detected from highest to lowest is as follows: Gamma fibrinogen (GAMMA) > heparan sulfate (HEP) > hyaluronan (HAS)> pregnancy associated plasma protein (PAP) > growth differentiation factor 15 (G15) > plasminogen activator inhibitor (PAI) > syndecan-1 (SDC)

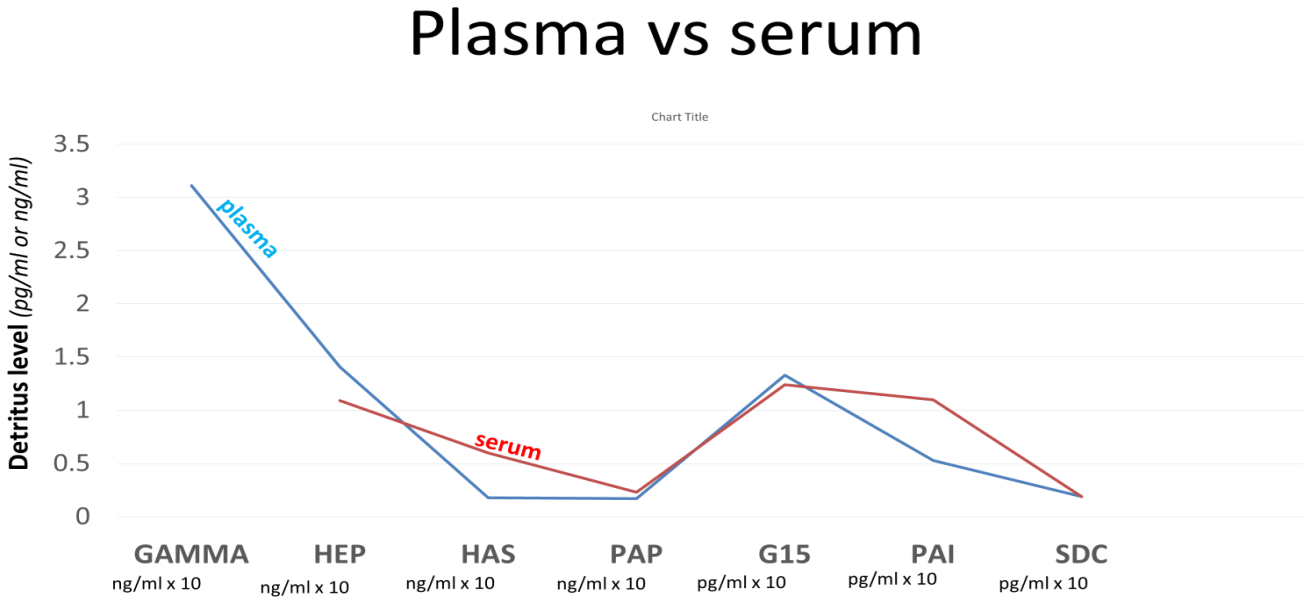

Figure 12. plasma vs serum: line graph

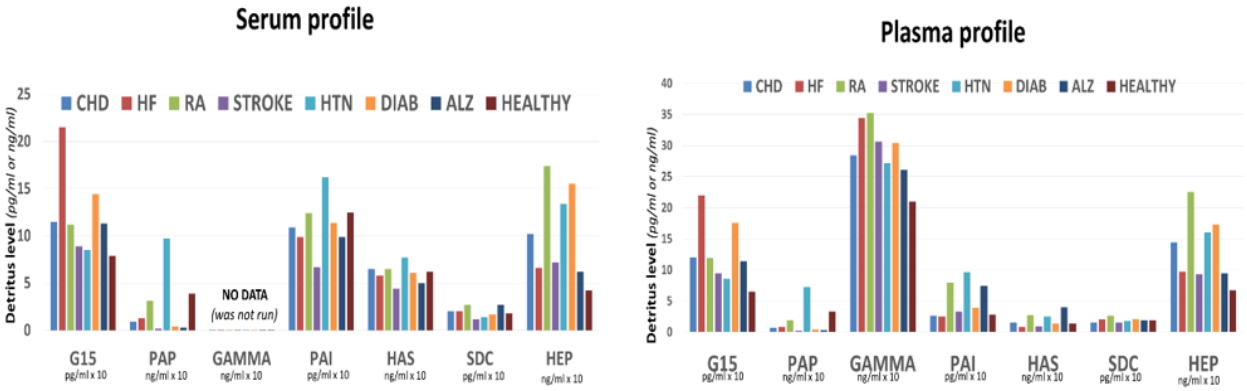

Figure 13. Plasma vs serum: bar graph

3. GDF as a viable fingerprint across chronic disease spectrum:

a) **Characteristic fingerprint for each disease:**

Each disease showed a unique 'fingerprint', which confirms the effectiveness of the GDF as a tool for diagnosing a wide spectrum of chronic diseases

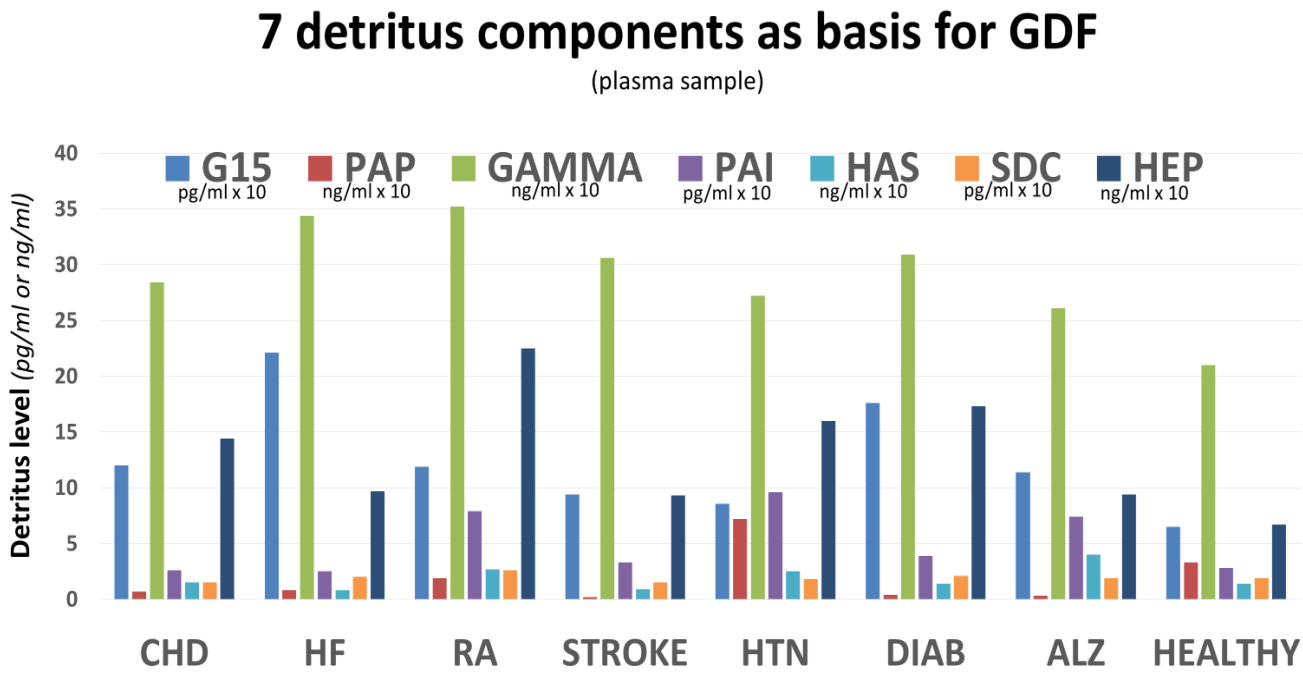

Figure 14. distinct fingerprint across spectrum of chronic diseases

b) Utility of a fingerprint platform:

In perspective, GDF comprises of biological materials like the biological nature of DNA. In DNA fingerprinting, fragments of DNA are separated on a gel using a technique called electrophoresis, while GDF involves analysis and quantitation of gycocalyx detritus shed in the blood stream per ELISA (Fig. 15). The usefulness of each diagnostic system depends on a robust library of fingerprints for comparison with unknown samples.

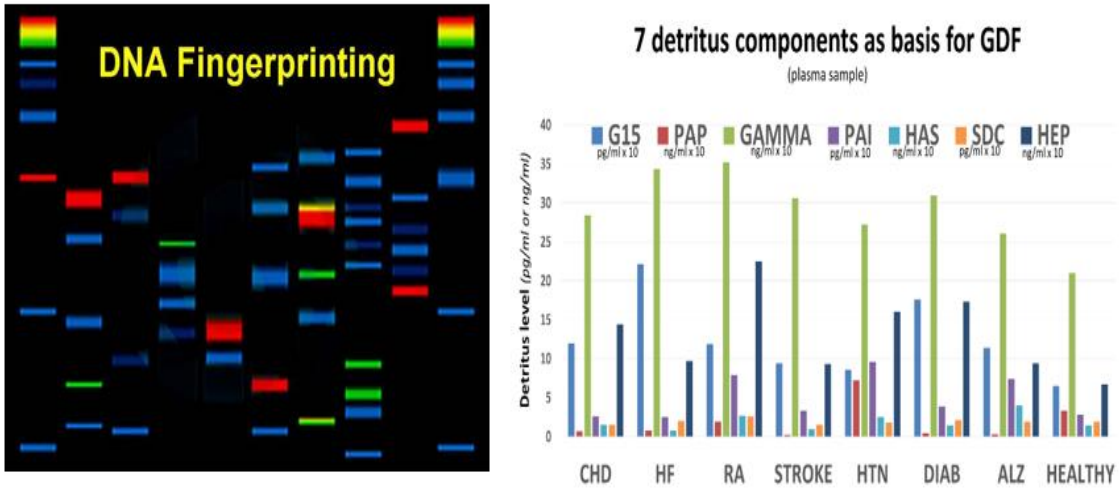

Figure 15. GDF is based on the analysis and quantitation of gycocalyx detritus shed in the blood stream per ELISA technique, while DNA fingerprint is based on fragments of DNA analyzed by gel electrophoresis,

c) Discussion:

Gamma fibrinogen is the most sensitive of the 7-biomarker panel. The disruption of blood flow creates transient clotting, which is detected by this biomarker. The next sensitive biomarker is heparan; 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, the hyaluronan, a glyocosaminoglycan that abuts the heparan sulfate starts to shed, which is the 3rd most sensitive biomarker. In the process of heparan and hyaluronan disruption is intrusion to the membrane, which activates the proteolytic enzyme called pregnancy-associated plasma protein A (PAPP-A), to start a healing or repair process (particularly elevated with imminent plaque disruption). PAPP-A shows to be the 4th most sensitive marker. Another repair protein is the growth differentiation factor 15 (G15), which shows as the 5th marker to appear. If these repair proteins are do not contain disruption, the integrity of the endothelial glycocalyx becomes compromised triggering fibrinolysis or hemorrhaging. As a further defense, the endothelium releases an embedded protein called plasminogen activator inhibitor-1 (PAI-1); this protein

mainly produced by the endothelium suppress fibrinolysis or hemorrhaging; this shows up as the 6th biomarker release. The last marker for membrane disruption is syndecan -1 (Syn-1), which is found at the inner core of the glycocalyx to maintain cytoskeletal integrity. This is the least level of biomarker detected in the blood stream, which appears to be released as a vestige of the membrane.

Indeed, these biomarkers are detected at any stage of the disease cycle and have been used individually or as single markers in the clinic with limited success. While there is a sequential rationale for the shedding of these biomarkers, disease is a dynamic process, and a mixture of these biomarkers are present in the milieu at any given time reflecting the stage of the disease or type of disease. On the other hand, healthy normal shedding of cells releases background levels of detritus, which and if taken individually could be misconstrued to be associated to a certain disease.

4. Development path for pattern recognition

This involves machine learning algorithm, which classifies data based on statistical information extracted from patterns. Building algorithms include multiple analysis of variance (MANOVA) to test the significance of each of the dependent variables separately and establishes causality between and amongst the biomarkers and diseases.

As the data set expands within the Arterez Disease Repository database, this will contain various stages of diseases within each person, as a reference to properly identify, diagnose, and treat multiple chronic diseases *(Fig. 16).*

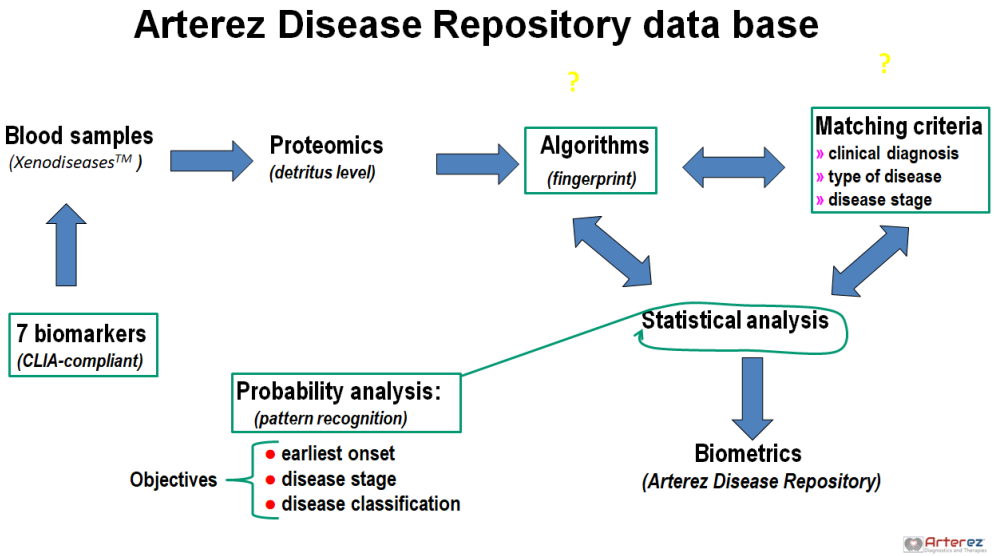

Figure 16. Development pathway and establishment of an Arterez Disease Diagnostic Repository data base

5. Clinical application for GDF

a). Early onset diagnosis

The Glycocalyx Detritus fingerprint™ proved to be effective in creating distinct patterns or fingerprints of the different family members of CVD tested and other chronic diseases. The glycocalyx detritus fingerprint™ is the first analytical tool to diagnose diseases, to assist in targeted treatment. If a disease is detected early when there is no severe damage to the organs, the disease would be easier to treat without any complications. Currently, there is no practical orfeasible way of monitoring glycocalyx disruption. *(Fig 17).*

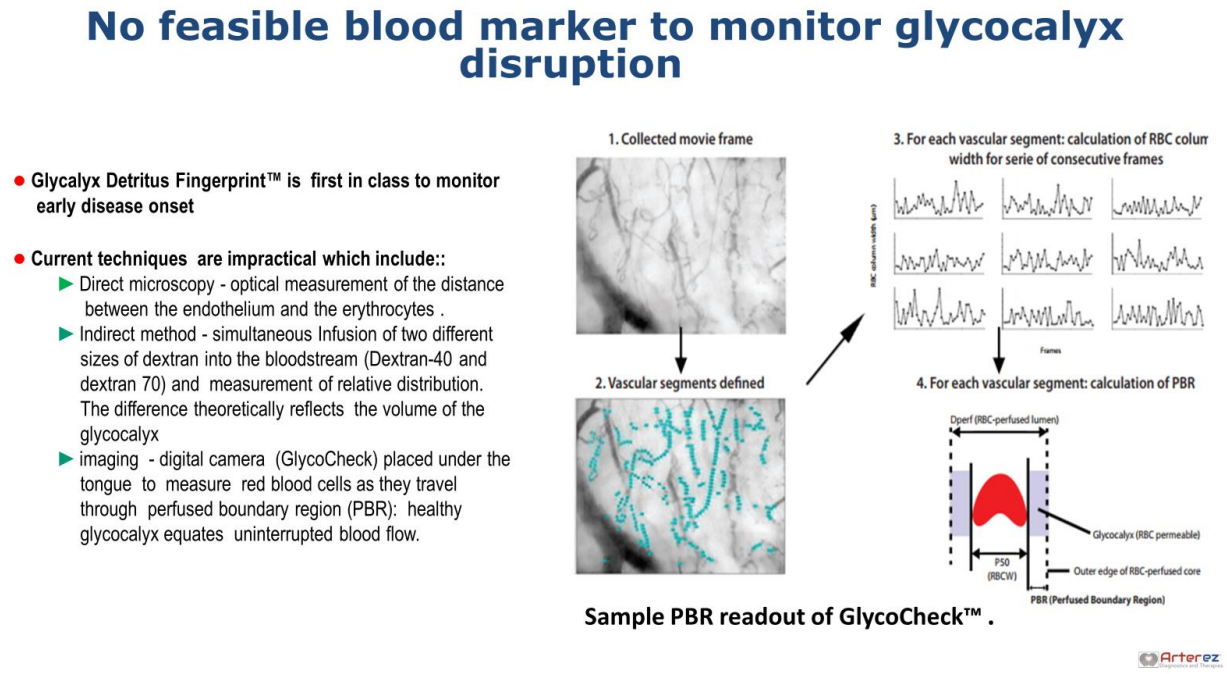

Figure 17. GDF is the first of its kind to offer a practical detection of the early onset of diseases

b). Diagnosis guide for therapy

The Glycocalyx Detritus fingerprint™ can be used twofold: 1) as a companion diagnostic for custom therapies (e.g., Embotricin™), or 2) 'stand-alone' diagnostic to monitor or evaluate the traditional symptom-targeted therapies *(Fig 18).*

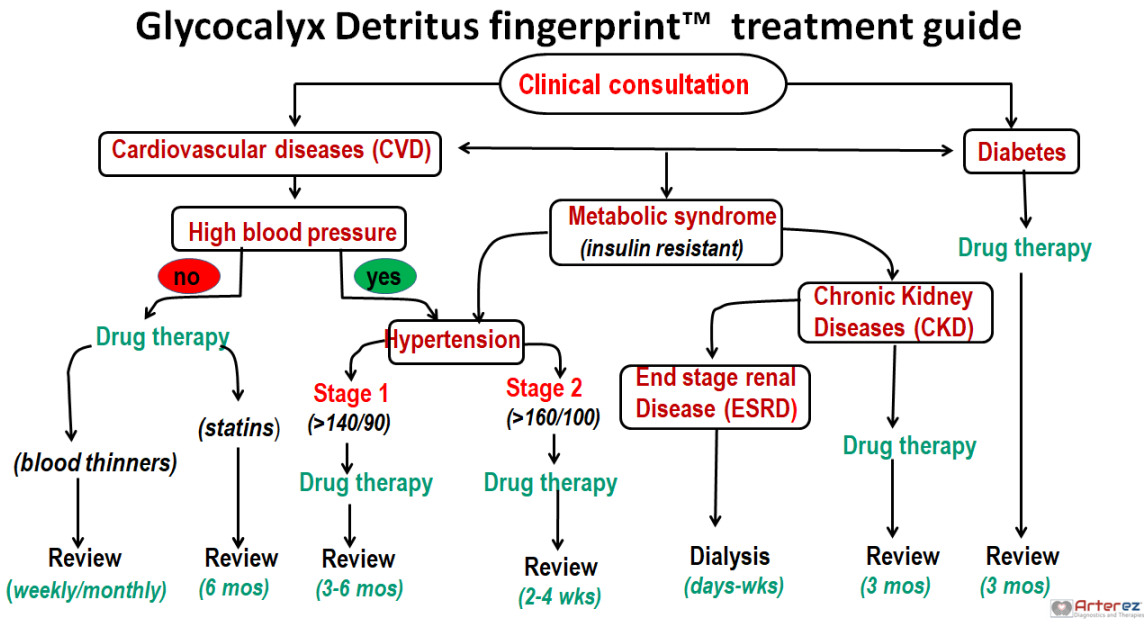

Figure 18. Scheduled use of the Glycocalyx Detritus fingerprint™ to monitor Embotricin™ or traditional symptom-targeted therapies