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(54) Title: DIAGNOSIS OF DIABETES BY DETECTING AGGREGATED AMYLIN IN ERYTHROCYTES

FIGURES

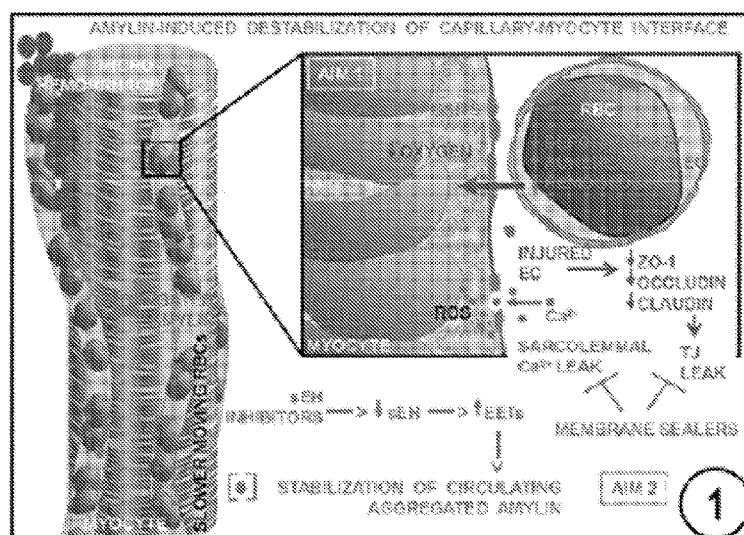


FIG. 1

(57) Abstract: The presently-disclosed subject matter generally relates to methods for detecting the presence of amylin in a patient's erythrocytes, determining the risk for the development of prediabetes, type-2 diabetes or comorbidities thereof, and methods of treating said diseases and risks.

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DIAGNOSIS OF DIABETES BY DETECTING AGGREGATED AMYLIN IN ERYTHROCYTES

GOVERNMENT INTEREST

[0001] This invention was made with government support under grant numbers R01HL118474, R01AG053999, R01HL135000, and R01AG057290 awarded by the National Institutes of Health. The government has certain rights in the invention.

RELATED APPLICATIONS

[0002] This application claims priority from United States Provisional Patent Application No. 62/767,131 filed on November 14, 2018, and United States Provisional Patent Application No. 62/883,992 filed on August 7, 2019 the entire disclosures of which are incorporated herein by this reference.

TECHNICAL FIELD

[0003] The presently-disclosed subject matter generally relates to methods for detecting the presence of amylin in a patient's erythrocytes, determining risk for the development of type II diabetes and comorbidities thereof, and determining effectiveness of treatments for patients at risk for developing type II diabetes and comorbidities thereof. The presently-disclosed subject matter also relates to methods for diagnosing and treating a subject with diseases related to the dysregulation of amylin including heart failure, cancer, stroke, amylin related renal hypoxia, amylin related brain hypoxia, amylin related cardiac hypoxia, microvascular disease, or age-related diseases.

BACKGROUND

[0004] Risk of diabetic heart failure (HF) begins years before type-2 diabetes (T2D) is clinically diagnosed and is higher in people with insulin resistance¹⁻⁵. Thus, it is becoming critically important to define early links between HF and T2D and to validate therapeutic targets. Amylin is a pancreatic β -cell hormone co-secreted with insulin²³, plays a role in normal glucose homeostasis^{27,28}, but it also is amyloidogenic and aggregates quickly when hypersecreted²³. Conditions associated with hypersecretion of amylin include obesity^{29,30}, pre-diabetic insulin resistance state^{29,30} and early phase of type-1 diabetes³¹. Aggregated amylin is known to cause apoptosis^{11,32}, oxidative stress^{33,34} and inflammation^{35,36} in the

pancreatic islets, thus contributing to the development of T2D in humans. The inventors found that aggregated amylin is abundantly present in failing hearts of patients with T2D or obesity, but not in controls^{6,7}. Recent efforts¹⁵⁻²² from several labs¹⁵⁻²² have confirmed that amylin aggregation is abnormally increased in brains¹⁵⁻²¹ of patients with T2D and dementia and in failing kidneys²² of individuals with T2D. The inventors^{6-9,16-18,25,26} (and others^{14,32,37-39}) showed that rats that overexpress human amylin (3-fold) in the pancreas (HIP rats) develop multiple organ impairments, including heart dysfunction^{6-10,25,26}. These impairments are associated with accumulation of aggregated amylin intracellularly. While the inventors interpret these findings to generally support the idea that therapies targeting hyperamylinemia will have a role in diabetes care, clearing aggregated amylin from cells has proven difficult. The scientific premise of the invention is based on accumulating evidence^{6-9,11-13,15,17-24,35} showing a role of aggregated amylin in target organ toxicity in humans, the presence of aggregated amylin in the blood of patients with type-2 diabetes⁸, *iii*) data showing that circulating aggregated amylin induces multiple organ impairments in HIP rats^{6-9,16-18,25,26,32,38} and mice³⁷⁻³⁹, *iv*) challenges to block amylin aggregation within the pancreatic secretory pathway²³, and *v*) lack of therapies directed to limit the uptake of circulating aggregated amylin or/and its deleterious effects in the heart.

[0005] Because hyperamylinemia coincides with hyperinsulinemia⁴⁰, cardiac accumulation of aggregated amylin might be a catalyst of the myopathic response in the pre-diabetic insulin resistance state. Data herein indicates that *i*) circulating aggregated amylin provokes myocardial microhemorrhages, and *ii*) the blood level of aggregated amylin increases with the transition from normal glucose homeostasis to impaired glucose tolerance and to overt hyperglycemia. The findings indicate that the interplay between pre-diabetes and hyperamylinemia creates a feed forward pathologic circuit that amplifies cardiac injury by amylin induced disruption of the capillary-myocyte interface (**Fig 1**).

[0006] Healthy RBCs are flexible and oval biconcave disk shaped, which facilitates the release of O₂ while squeezing through the capillaries. Slower moving RBCs result in hypoxia⁴¹⁻⁴⁴.

[0007] Detection of cardiac amylin deposition. Proteinaceous deposits immunoreactive for amylin are abundant in myocardial tissues from patients with HF and T2D or obesity, but not in non-failing hearts from lean individuals without T2D (controls)^{6,7}. Amylin-positive deposits were identified in blood vessel walls⁷, perivascular areas^{6,7}, myocardial interspaces and inside cardiac myocytes^{6,7}. The source of amylin deposited in the heart originates in the

pancreas, as no amylin mRNA was found in cardiac tissue or other extra-pancreatic tissues^{7,15}. To unambiguously validate the myocardial accumulation of amylin, reverse-phase high performance liquid chromatography (HPLC) and liquid chromatography tandem mass spectrometry (LC-MS/MS) was employed⁸. Based on the retention time of the standard amylin peptide as derived from the HPLC chromatogram, fractions of myocardial tissue lysates likely to contain amylin were collected and immunoblotted for amylin identification. Fractions positive for amylin by Western blot were then further tested by LC-MS/MS. The results convincingly demonstrated that amylin is contained in myocardium in patients with T2D, but not in non-failing hearts from lean subjects without T2D.⁸

[0008] Cardiac phenotypes associated with aggregated amylin. Amylin from rodents has a decreased propensity to aggregate, which was attributed to proline substitutions at positions 25, 28, and 29²⁴. Thus, the innate distinctions between human amylin and rodent amylin species was exploited to assess mechanistically the impact of a “human” hyperamylinemia on cardiac myocytes in a rat model transgenic for human amylin⁶⁻⁹. Human amylin-expressing rats were previously used to study complications of T2D in the cardiovascular^{6-9,25,26} and central nervous systems¹⁶⁻¹⁸. **TABLE 1** summarizes phenotypic changes in HIP rats. HIP rats have elevated blood levels of aggregated amylin^{7,8,16,18} and develop a late-onset T2D-like pathology³². Hyperamylinemia is associated with *a*) cardiac hypertrophy^{6,7} (via activation of Ca²⁺-mediated hypertrophy/remodeling signaling), *b*) diastolic dysfunction⁶ (via SERCA downregulation), *c*) arrhythmias^{25,26} (via increased CaMKII activity²⁵ and altered myocyte Na⁺²⁶), *d*) inflammation⁸ (via increased synthesis of IL-1 β ⁸), and *e*) neurologic deficits¹⁶⁻¹⁸ (via neuroinflammation¹⁶, impaired catecholamine synthesis⁹, and small blood vessel disease¹⁸). Confocal microscopy analysis of cardiac tissues from patients with T2D and HF demonstrated the presence of sub-sarcolemmal deposits of amylin⁸. Areas of myocyte amylin deposition were positive for accumulation of reactive aldehydes (4-HNE and MDA), indicating peroxidative damage of membrane lipids. In contrast, amylin-4-HNE/MDA adducts were undetectable in hearts from the control group. Pancreatic tissue (the positive control for amylin deposition) from a diabetic patient also showed amylin-4-HNE adducts, consistent with the role of aggregated amylin in inducing oxidative stress in the pancreas^{33,34}. Cardiac tissues from patients with diabetes or obesity showed also IL-1 β immunoreactivity in amylin-positive areas⁸. Consistent with findings in human tissues, HIP rat hearts showed sub-sarcolemmal accumulation of aggregated amylin, formation of amylin-4-HNE complexes and increased synthesis of IL-1 β ⁸. These results were mirrored in Langendorff perfused hearts and normal

mice intravenously injected with aggregated human amylin, but not in hyperglycemic rats that secrete wild-type (non-amyloidogenic) rat amylin⁸. Thus, exacerbated synthesis of IL-1 β is revealed as an unexpected, but critical stress-activated signaling pathway in response to the interaction of aggregated amylin with myocytes. These results⁸ are consistent with the demonstrated role of aggregated amylin in inducing IL-1 β release in pancreatic islets^{35,36}. In HIP rats, cardiac amylin accumulation provoked sarcolemmal Ca²⁺ leak⁶, which increased cytosolic Ca²⁺. In turn, the increase of cytosolic Ca²⁺ triggered the activation Ca²⁺-mediated hypertrophy signaling pathways (CaMKII-HDAC and calcineurin-NFAT). Myocyte Ca²⁺ dysregulation and pathologic Ca²⁺ signaling were replicated in isolated cardiac myocytes incubated with aggregated amylin⁶. These results may explain SERCA downregulation and diastolic dysfunction in HIP rats⁶.

Cardiac function/Structure Alteration	ROI
Amylin deposition	6
Diastolic dysfunction	6
Eccentric hypertrophy	7
Dilation	7
Arrhythmia	25
Myocyte Ca ²⁺ dysregulation	6
Elevated myocyte [Ca ²⁺]	26
Impaired protein biosynthesis	9
ROS production	8
Sarcolemmal lipid peroxidation	8
Inflammation	8
Cardioprotection by reducing systemic amylin dyshomeostasis	7
Brain function/Structure Alteration	ROI
Amylin deposition	16
Learning & memory deficits	16,18
Vestibulomotor dysfunction	16,18
Microhemorrhages	18
White matter rarefaction	18
Blood brain barrier injury	17

TABLE 1: PHENOTYPIC CHANGES IN DIABETIC RATS EXPRESSING HUMAN AMYLIN IN THE PANCREAS

[0009] *Sex differences.* Female HIP rats develop hyperglycemia and neurologic deficits later in life compared to males (i.e., ~12 months vs. ~18 months of age)¹⁸. To test further a possible sex difference in amylin pathology, amylin knockout (AKO) male and female mice which were intravenously infused with either human amylin (i.e., the aggregated form) or non-amyloidogenic (i.e., monomeric) rodent amylin (2 pg/g body weight, q.d., 7 days) were

tested¹⁰. AKO mice infused with human, but not rodent amylin, showed amylin deposits in the myocardium. Cardiac amylin level was larger in males compared to females, indicating a sex-dependent effect which might be estrogen-dependent. These results¹⁰ are consistent with previous data⁵¹ indicating that pancreatic amylin deposition is more abundant in men compared to women. This sex difference in amylin pathology was attributed to increased insulin resistance in men^{52,53}. Ovariectomy or employing older female mice (as in HIP rats¹⁸) might mitigate the sex difference in amylin-induced pathology. Sarcolemmal Ca²⁺ leak and Ca²⁺ transients were increased in myocytes isolated from male AKO mice infused with human amylin while no significant changes occurred in either females injected with human amylin or in rat amylin-infused mice¹⁰. Indeed, previous studies demonstrated sex differences in cardiac myocyte ion channels⁵⁴⁻⁵⁸, Ca²⁺ cycling^{59,60}, contractions⁵⁹ and metabolism^{61,62}, which may contribute to the differential effects of amylin stress. These results¹⁰ indicate that aggregated amylin accumulates preferentially in male vs. females and is an independent contributing mechanism to myocyte Ca²⁺ dysregulation in T2D.

[0010] EETs are formed by endothelial cells⁴⁵⁻⁴⁷ and have demonstrated ability to reduce proteinaceous deposition on blood vessel walls^{48,49}. Endogenous levels of EETs were elevated in *pre-diabetic* HIP rats⁷ by treatment with an inhibitor of Soluble epoxide hydrolase (sEH), the enzyme that degrades EETs⁴⁵. Rats in the treatment group showed lower amylin incorporation into RBCs and cardiac myocytes and improved heart function. Using a sEH inhibitor to increase the levels of EETs in *diabetic* HIP rats proved less efficient⁷.

[0011] Oxygen is essential for cell function and cell survival. RBCs deliver oxygen to cells and tissues via mechanisms that involve the passage of RBCs through capillaries. This process is enabled by the viscoelastic properties of the RBCs, which allow them to be deformed within capillaries. In type-2 diabetes, the oxygen-carrying capacity of RBCs and the integrity and stability of the capillaries decline, exacerbating the risk of tissue hypoxia and end organ malfunction. The underlying mechanisms are complex and incompletely understood.

[0012] Physiological responses to low oxygen levels are primarily driven by the stabilization of the α subunits of the *hypoxia-inducible transcription factors* HIF-1 and 2. HIF-2 regulates the hypoxia response by elevating the renal expression of EPO, a hormone that signals an increased demand for RBCs from the bone marrow, which then increases the production of RBCs. Stabilization of HIF-2 α also induces *arginase* expression in vascular endothelial cells. Because arginase has the same substrate (L-arginine) as nitric oxide (NO)

synthase, increased *arginase* production/ activation may reduce NO availability. Depleted NO production impairs relaxation of the blood vessels and affects microvascular autoregulation. Thus, increased EPO coupled with arginase-NO dysregulation constitutes one of the multiple molecular derangements linking systemic hypoxia with microvascular dysfunction.

[0013] Metabolic derangements that occur before the development of overt hyperglycemia may induce microvascular dysfunction. In prediabetes, pancreatic β -cells compensate for insulin resistance by increasing the secretion of insulin (hyperinsulinemia). Amylin (also known as islet amyloid polypeptide; IAPP), is a 37-amino acid peptide synthesized and co-secreted with insulin in response to physiological stimuli. It is normally soluble, crosses the blood brain barrier and binds to neurons in the feeding centers participating in the regulation of gastric fluxes. Amylin from humans and a few other species, including cats, dogs and monkeys, but not rodents, has an increased propensity to aggregate, forming amyloid (i.e., amylin dyshomeostasis). This triggers β -cell apoptosis by mechanisms involving incorporation of aggregated amylin into cellular membranes. Amylin deposition is present in failing hearts and kidneys of patients with type-2 diabetes and in brains of humans with dementia. Rats develop type-2 diabetes linked to amylin dyshomeostasis (i.e. the HIP rat model for type-2 diabetes) develop heart dysfunction and neurological deficits sooner than age- and blood glucose-matched rats that develop type-2 diabetes in the absence of amylin dyshomeostasis (i.e. the UCD rat model for type-2 diabetes). The upregulation of epoxyeicosatrienoic acids (EETs) in endothelial cells appeared to protect against cardiac amylin accumulation in HIP rats, which correlated with improved heart function. The impact of elevated blood levels of amylin on blood cells and the microvasculature remains unknown.

[0014] The hypothesis that systemic amylin dyshomeostasis alters the interaction between RBCs and capillaries leading to hypoxic-ischemic tissue injury was tested. To test this hypothesis, the amylin levels in RBCs from humans with and without diabetes, and used transgenic rats, RBC transfusions and pharmacological tools for mechanistic studies was measured.

[0015] Human Amylin and human A β polyclonal antibodies to develop the detection of Amylin and A β along with their oligomers were developed in diseased and normal condition. Rabbit has been used as a host to generate the antibodies. First, a bioinformatics analysis was carried out to determine the most immunogenic regions in Amylin and A β peptides. N-terminal and C-terminal regions from both Amylin and A β were used as an immunogen to

generate the antibodies. Generated antibodies were used in ELISA to check the titer. The 1:100000 titer for each antibody worked. These antibodies were successfully detected the native protein in Immunohistochemistry. These antibodies were used in western blot where A β antibody was detecting monomer. These antibodies specifically detect the native form of human Amylin peptides.

SUMMARY

[0016] The presently-disclosed subject matter meets some or all of the above-identified needs, as will become evident to those of ordinary skill in the art after a study of information provided in this document.

[0017] This Summary describes several embodiments of the presently-disclosed subject matter, and in many cases lists variations and permutations of these embodiments. This Summary is merely exemplary of the numerous and varied embodiments. Mention of one or more representative features of a given embodiment is likewise exemplary. Such an embodiment can typically exist with or without the feature(s) mentioned; likewise, those features can be applied to other embodiments of the presently-disclosed subject matter, whether listed in this Summary or not. To avoid excessive repetition, this Summary does not list or suggest all possible combinations of such features.

[0018] The presently-disclosed subject matter generally relates to methods for detecting the presence of amylin in a patient's erythrocytes, determining risk for the development of type-2 diabetes, and determining effectiveness of treatments for patients at risk for developing type-2 diabetes and comorbidities thereof.

[0019] The present invention also relates to a method of detecting amylin in a patient, said method comprising: obtaining a blood sample from a human patient; detecting whether amylin is present in the erythrocytes by conducting an amylin ELISA; and using the amylin ELISA to determine the amount of amylin in the patient's erythrocytes. In some embodiments of the present invention, the patient is treated by increasing circulating Epoxyeicosatrienoic acids (EETs) if the amount of amylin in the patient's erythrocytes is higher than the amount of amylin in healthy control erythrocytes. In further embodiments of the present invention, the method also includes diagnosing the patient with pre-diabetes if the amount of amylin in the patient's erythrocytes is higher than the amount of amylin in healthy control erythrocytes and lower than the amount of amylin in diabetic control erythrocytes;

and treating the patient with lifestyle changes, biological, genetic, or pharmacological intervention suitable for the treatment of pre-diabetes. In other embodiments of the present invention, the patient is diagnosed with pre-diabetes when the amount of amylin in the erythrocytes is between about 1 ng/g and about 2 ng/g total protein.

[0020] Other embodiments of the present invention include a method for diagnosing the patient with type-2 diabetes, if the amount of amylin in the patient's erythrocytes is higher than the amount of amylin in healthy control erythrocytes and pre-diabetic control erythrocytes; and treating the patient with therapeutically effective amount of an anti-diabetic therapeutic. In further embodiments, the patient is diagnosed with type-2 diabetes when the amount of amylin in the erythrocytes is greater than or equal to about 2 ng/g total protein.

[0021] In some embodiments of the present invention, a patient is diagnosed as at risk for developing a comorbidity of type-2 diabetes, if the amount of amylin in the patient's erythrocytes is higher than the amount of amylin in healthy control erythrocytes; and treating the patient by increasing circulating Epoxyeicosatrienoic acids (EETs). In further embodiments, circulating EETs are increased by administering an effective amount of an inhibitor of soluble epoxide hydrolase. In other embodiments, the patient is diagnosed with a comorbidity of type-2 diabetes when the amount of amylin in the erythrocytes is about 1.7 ng/g total protein or greater. In some embodiments of the present invention, comorbidities of type-2 diabetes is cancer, heart failure, or stroke. In further embodiments, the patient is treated with an effective amount of an anti-cancer therapeutic, a heart failure therapeutic, or a therapeutic for the treatment of stroke.

[0022] Also described herein is a method of diagnosing the patient with amylin related hypoxia if the amount of aggregated amylin in the patient's erythrocytes is higher than the amount of aggregated amylin in healthy control erythrocytes; and treating the patient by increasing circulating Epoxyeicosatrienoic acids (EETs). In some embodiments of the present invention, the amylin related hypoxia is renal, cardiac, or brain hypoxia. In further embodiments of the present invention, circulating EETs are increased by administering an effective amount of an inhibitor of Soluble epoxide hydrolase.

[0023] One embodiment of the present invention is a method to determine the effectiveness of biologic, lifestyle changes, or pharmaceutical intervention in preventing complications in a patient with type-2 diabetes or co-morbidity thereof, or amylin related renal hypoxia, said method comprising: obtaining a first blood sample from the patient

diagnosed with type-2 diabetes or co-morbidity thereof, or amylin related renal hypoxia; using an aggregated amylin ELISA to determine the amount of aggregated amylin in the patient's erythrocytes; administering an effective amount of biologic, lifestyle change, or pharmacologic intervention; obtaining a subsequent blood sample from the patient; using the aggregated amylin ELISA to determine the amount of aggregated amylin in the patient's erythrocytes; and determining the intervention is effective if the amount of aggregated amylin in the erythrocytes in the subsequent sample is less than the amount of aggregated amylin in the erythrocytes in the first sample.

[0024] In many embodiments of the present invention, the treatment increases circulating Epoxyeicosatrienoic acids (EETs).

BRIEF DESCRIPTION OF THE DRAWINGS

[0025] **FIG. 1.** shows schematic illustrating the amylin-induced destabilization of capillary-myocyte interface.

[0026] **FIGs. 2A-C.** show analysis of amylin in matched plasma, Red Blood Cells, Myocytes, and White Blood Cells in humans presenting with obesity and/or heart failure or non-obese. A) Western blot analysis of amylin in matched plasma, white blood cells (WBCs) and red blood cells (RBCs) from a patient with obesity ($BMI \geq 30$) and HF. B) Comparative analysis in paired RBCs and cardiac myocytes from the same patients. C) The relationship between RBC amylin levels, obesity and HF.

[0027] **FIGs. 3A-C.** show characterization of AKO rats, amylin expression, glucose homeostasis, and body weight. Demonstration of lack of amylin expression in AKO rats. B) Deletion of amylin gene (AKO rats) reduced the difference in glucose homeostasis between male and female rats. C) Body weight in AKO vs. WT rats.

[0028] **FIGs. 4A-E.** show characterization of amylin in RBCs from HIP rats that are healthy, prediabetic, or diabetic. Amylin in RBCs from HIP rats with normal blood glucose (healthy), prediabetes (PD) or diabetes (D) measured by flow cytometry analysis A), western blot (cropped gel; B) and ELISA C). Cell shape changed in RBCs from HIP v. WT rats and in WT rat RBCs incubated with human amylin (50 μM) for 2 hours, as measured by flow cytometry (PCD – Pearson coefficient of dissymmetry; D). The total amount of hemoglobin in 100 μL RBCs suspension measured spectroscopically was lower in HIP v. WT rats E).

[0029] FIGs. 5A-C. show characterization of amylin in cardiac tissue of rats. Confocal microscopy analysis of amylin in cardiac tissue from WT rats infused with HIP rat RBCs v. WT rat RBCs. B) Immunostaining for amylin, glycophorin A (a protein abundant in RBCs) and collagen IV in cardiac tissue isolated from AKO rats injected with HIP rat RBCs. Scale bar, 20 μ m. C) Adhesion to cultured endothelial cells was compared for RBCs from HIP v. WT rats and for RBCs from WT rats $\pm$ incubation with human amylin (50 μ M) for 2 hours.

[0030] FIG. 6. shows characterization of plasma EPO levels from healthy HIP rats and age-matched HIP diabetic rats.

[0031] FIG. 7. shows glucose tolerance of WT and HIP rats fed a normal or high fat diet.

[0032] FIGs. 8A-C. show Arginase 1 & 2 A) expression, B) protein levels, and C) activity in cardiac tissue from WT and HIP rats.

[0033] FIGs. 9A-E. show structural integrity and stability of myocardial capillaries. Prussian blue staining showed microhemorrhages in myocardial tissue from a 15 mo. old HIP rat, but not in an age matched WT rat. (B, C) Same as above in WT rats infused with HIP rat RBCs v. WT rat RBCs; scale bar, 20 μ m. D) Western blot analysis of tight junction proteins in HIP v. WT rats. E) Amylin in myocardial vcapillaries isolated from HIP v. WT rats (ELISA).

[0034] FIGs. 10A-C. show structural integrity and stability of myocardial cells. A) Co-staining for amylin and collagen IV in a 15 mo old HIP rat v. age matched WT rat. Same as in above form amylin and IBA-1, in B) and for endothelial cells, in C. Scale bar, 20 μ m in A); 100 μ m in (B&C).

[0035] FIGs. 11A-C. show infusion of WT or HIP rats with WT or HIP rat RBCs and detection of amylin in cells and tissues. A) Western blot analysis of amylin in plasma, RBC lysates and myocardial capillaries from WT rats infused with HIP rat RBCs v. WT rat RBCs B) Co-staining for amylin (brown) and collagen (green) in cardiac tissues from WT rats infused with HIP rat RBCs v. WT rat RBCs. C) same as in above for amylin (brown) and IBA-1 (green). Scale bar, 20 μ m.

[0036] FIG. 12. shows Sarcolemmal and cytosolic fractions of myocytes.

[0037] **FIGs. 13A-B.** show increased RyR oxidation in WT and HIP hearts. A) Measurement of free thiols contained in RyRs. B) Percentage of RYR free thiols calculated from experiments as in panel A.

[0038] **FIG. 14.** shows Amylin aggregation in serum in the absence or presence of added glucose.

[0039] **FIGs. 15A-B.** show effect of 14, 15-EET on amylin aggregation in myocytes.

[0040] **FIGs. 16A-C.** show effect of membrane sealant P188 on amylin-induced increase in sarcolemmal Ca^{2+} leak (A-B) and Ca^{2+} transient amplitude (C).

[0041] **FIG. 17.** shows the effect of P188 on lipid peroxidation in HIP rat myocytes.

[0042] **FIGs. 18A-I** show amylin-coated RBCs in human pathology. (a) Representative Western blots showing the amylin monomer in matched plasma, RBC and white blood cell (WBC) lysates from an individual with type-2 diabetes. Recombinant human amylin served as control. (b) Whisker box plots comparing the concentration of amylin, measured by ELISA, in RBCs from healthy individuals (h; dark green; n=66) versus individuals with various diseases or combination thereof, including type-2 diabetes (T2D; blue; n=69), heart failure (HF) with diabetes (HF-T2D; dark red; n=49) and without diabetes (HF w/o T2D; light red; n=59), cancer with diabetes (C-T2D; black; n=33) and without diabetes (C; gray; n=58), and stroke (S; yellow; n=13). RBC lysates from patients with type 1 diabetes (T1D; black; n=5) are the negative control for amylin. Statistical significance of the differences in amylin level was assessed using One-way ANOVA with the Bonferroni post-test for comparing all pairs of columns. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. (d-j) The correlation between RBC amylin and HbA1c in groups of healthy (c) and diseased individuals (d-i) described in (b). Out-of axis amylin-HbA1c levels: 25.2-5.8 and 12.2-4.6 in (d); 16.3-5.5 in (f) and 10.7-5.3 and 13.1-5.1 in (i), respectively. The Spearman nonparametric correlation analysis was performed in GraphPad and the values for the Spearman r and P are indicated on the plots.

[0043] **FIGs. 19A-F** show accumulation of amyloid-forming human amylin in RBCs from diabetic rats. (a) Amylin concentration, measured by ELISA, in matched plasma and lysates of WBCs and RBCs from HIP rats and WT littermates (n=4/group). (b) Representative flow cytometry graphs (upper) and mean intensity (lower) for amylin/Alexa Fluor 488 fluorescence in RBCs from healthy, prediabetic and diabetic HIP rats (n=5 rats/group). (c) Amylin concentration in RBC lysates from 16 months old WT rats (n=7) and

healthy (n=6), prediabetic (n=15) and diabetic (n=16) HIP rats measured by ELISA. (d and e) Representative images of co-staining for amylin and hemoglobin (d) and amylin and glycophorin A (e) in RBCs from age-matched WT and diabetic HIP rats (n=3 rats/group). Scale bar, 10 μ m (top row) and 5 μ m (middle and bottom rows). (f) Representative STORM images showing RBCs from HIP and WT rats stained for human amylin (red) and Glycophorin A (green). (Scale bar 2 μ m). (n=3 for each rat group). *P <0.05; **P <0.01 by t-test (a) and One-way ANOVA (b and c).

[0044] FIGs. 20A-H show pathophysiological changes induced by amyloid-forming amylin in RBCs. (a) Hemoglobin levels in RBCs from WT, HIP and UCD rats (n=11 rats/group). (b) Oxygen dissociation curve for RBCs from WT, HIP and UCD rats (n=5 rats/group). (c) Representative example of cell shape distribution of RBCs from WT (orange), UCD (blue) and HIP (pink) rats (n=6 rats/group) and the Pearson coefficient of dissymmetry (PCD) calculated from these data. (d) Percentage of RBC hemolysis in hypo-osmotic solutions. The NaCl concentration for 50% hemolysis of WT, HIP and UCD RBCs is indicated in the inset. (n=4 rats/group). (e and f) Representative images of co-staining for amylin and collagen IV (Col IV) in kidney tissue sections showing amylin deposition in arterioles and interstitial tissue (e) and in the glomerulus (f) in diabetic HIP rats but not in diabetic UCD rats (n=3 rats/group). (g) Representative images of co-staining for amylin and ionized calcium binding adaptor molecule 1 (IBA1) in kidney tissue sections from diabetic HIP and diabetic UCD rats (n=3 rats/group). (h) Representative images of staining for the cluster of differentiation 68 (CD68; ED1) in kidney tissue section from diabetic HIP and diabetic UCD rats (n=3 rats/group). (Scale bar 50 μ m) *P<0.05; **P<0.01 by One-way ANOVA with Tukey's post-test (a and c).

[0045] FIGs. 21A-H show modulation of renal hypoxia markers by amylin dyshomeostasis. (a) EPO levels in plasma from 16 months WT rats (n=17), diabetic UCD rats (n=8) and diabetic HIP rats (n=17). (b) Percentage number of reticulocytes over total numbers of RBCs in blood of WT, HIP and UCD rats (n=3 for each rat group). (c) Hematocrit levels in diabetic HIP and UCD rats and WT controls (n=6 rats/group). (d-h) Protein levels of HIF-1 α (d), HIF-2 α (e), arginase-1 (f) and arginase-2 (g) and arginase activity (h) measured by ELISA in renal tissues from 16 months old WT rats and diabetic UCD and HIP rats (n=10 rats/group). *P <0.05; **P <0.01; ***P <0.001 by One-way ANOVA with Tukey's post-test.

[0046] **FIGs. 22A-G** show effect of increasing endogenous EETs on RBC-capillary coupling and renal hypoxia signaling. (a) Average numbers of RBCs adhered to culture vascular endothelial cells when fixed hematocrit of RBCs flowed over cultured EC at constant rate for constant time (n=3/ group). (b) Analysis of the adhesion of RBCs isolated from HIP rats and WT littermates to cultured vascular endothelial cells (n=8/group). (c) Adhesion of WT rat RBCs to cultured vascular endothelial cells with/without incubation with recombinant human amylin (50 μ M) for 2 hours (n=5/group). (d) Attachment of RBCs from WT and diabetic HIP rats to vascular endothelial cells in the absence or in the presence of various amounts of EETs (n=8 rats/group). (e) Representative images of staining for amylin and Col IV in kidney sections from diabetic HIP rats (HIP-UT) and diabetic HIP rats with pharmacologically upregulated EETs (HIP-T). The scatterplot shows the percentage of the tissue area that is positive for amylin (n=3 rats/group). (f-g) Protein levels of HIF-2 α (f) and HIF-1 α (g) in whole kidney tissue homogenate from rats in the HIP-UT and HIP-T groups (n=4 rats/group). *P <0.05; **P <0.01; ***P <0.001 by t-test.

[0047] **FIG. 23A-H** show altered oxygen sensing in kidneys following transfusion with amylin-loaded RBCs (a) Plasma EPO levels in WT rats transfused with RBCs from i) WT rats (n=7); ii) diabetic UCD rats (n=7) and iii) diabetic HIP rats (n=7). (b and c) Protein levels of HIF-1 α (b) and HIF-2 α (c) in renal tissue homogenate from the rats described in (a). (d) Representative images of amylin and glycophorin A co-staining (left panel) and of amylin and hemoglobin co-staining (right panel) in RBCs from UCD rats infused with aggregated human amylin (daily injection of 0.08 μ g/g body weight for 7 days; n=3 rats/group). (e) Plasma EPO levels in diabetic UCD rats (n=3) at baseline and at the end of the acute intravenous treatment with aggregated human amylin (hA). (f-g) Protein levels of HIF-2 α and HIF-1 α (f) and arginase-1 and arginase-2 (g) in renal tissue homogenates from diabetic UCD rats injected with human amylin versus non-injected diabetic UCD control rats (n=3 rats/group). (h) Cartoon illustrating the proposed mechanism through which “human” hyperamylinemia exacerbates hypoxia signaling in diabetic rats with pancreatic expression of non-amyloid forming amylin rat amylin (UCD rats). *P <0.05; **P <0.01; ***P <0.001 by One-way ANOVA with Tukey’s post-test (a-c) or two-tailed t-test (e-g).

[0048] **FIG. 24** shows Representative western blot analysis (*Left*) of high molecular weight amylin oligomers in plasma, RBC lysate and white blood cell (WBC) lysate from an individual with type-2 diabetes. (*Right*) The Ponceau S staining of the blot show in *Left* panel.

[0049] **FIG. 25** shows representative images of Thioflavin S (green) and amylin (red) staining in the pancreas from a diabetic HIP and a control WT rat (n=3/group). Scale bar, 30 μ m.

[0050] **FIG. 26** shows representative TEM images showing RBCs from HIP and WT rats, stained with human amylin primary antibody and gold (10nm) labelled secondary antibody. (Scale bar 1 μ m)

[0051] **FIG. 27** shows PCD for WT rat RBCs and WT rat RBCs incubated ex vivo with oligomerized human amylin (n=5 preparations/group).

[0052] **FIGs. 28A-C** show volume of urine excretion (A), albuminuria (B) and creatinine clearance rate (C) in 16 months old WT, diabetic UCD and diabetic HIP rats (n=6 rats/group). $^{**}P \leq 0.01$; $^{***}P \leq 0.001$ by One-way ANOVA.

[0053] **FIG. 29** shows gross spleen weights of 16 months old WT rats and diabetic HIP rats (n=10 spleens/group). Data are means $\pm$ SEM. $^{*}P < 0.05$

[0054] **FIG. 30** shows levels of von Hippel-Lindau (vHL) protein in the renal tissues from 16 months old WT rats and diabetic HIP rats (n=5 rats/group). Data are means $\pm$ SEM.

[0055] **FIG. 31** shows western blot and ELISA analyses for VCAM-1 expression in kidney capillaries lysates of WT and HIP rats (n= 3 rats/group).

[0056] **FIGs. 32A-B** show reduced amylin deposition in kidneys correlated with partially reduced imbalance of arginase expression and arginase activation in HIP rat kidney tissues. (n=4 rats/group). Data are means $\pm$ SEM. $^{*}P < 0.05$.

[0057] **FIGs. 33A-D** show ELISA showing higher titers in collected bleeds. A. ELISA of Amylin N-terminal, C-terminal and A β N-terminal, C-terminal antibody for 1st and 2nd bleed. B. ELISA of Amylin N-terminal, C-terminal and A β N-terminal, C-terminal antibody for 2nd and 3rd bleed. C. ELISA of Amylin N-terminal, C-terminal and A β N-terminal, C-terminal antibody for 3rd and 4th bleed. D. ELISA of Amylin N-terminal, C-terminal and A β N-terminal, C-terminal antibody for Final Bleed.

[0058] **FIGs. 34A-C** show a direct ELISA demonstrating purified antibodies are specific for their antigen. A. Direct ELISA of Amylin N-terminal and C-terminal antibody was performed on HIP rat pancreas and AKO rat pancreas. B. Direct Elisa of A β N-terminal and C-terminal antibody was performed on AD brain and wild type brain. C. Diagram of ELISA.

[0059] **FIG. 35** shows custom ELISA with antibodies showed similar results with commercially available ELISA. Left Panel. Direct Amylin ELISA with generated N-terminal antibody in plasma samples of HIP, WT and AKO mouse. Right Panel. Sandwich Amylin ELISA with commercially available kit in plasma samples of HIP, WT and AKO mouse.

[0060] **FIGs. 36A-B** show immunohistochemistry delineating how antibodies are specific for their proteins. A. Immunohistochemistry of HIP and AKO Rat pancreas with Amylin N-terminal and C-terminal antibody. B. Immunohistochemistry of AD rat brain and WT Rat heart with A β N-terminal antibody.

[0061] **FIGs. 37A-B** show A β N-terminal and C-terminal antibodies specifically detect the A β peptides in western blot. Western blot in AD brain, WT brain and synthetic peptide using A β N-terminal (A) and A β C-terminal antibody (B).

[0062] **FIGs. 38A-B** show the design and creation of antibodies against amylin and amyloid β peptide. A. The peptides used to generate antibodies against amylin and A β . B. Injection and bleed collection schedule.

[0063] While the disclosure is susceptible to various modifications and alternative forms, specific embodiments thereof have been shown by way of example in the drawings and are herein described below in detail. It should be understood, however, that the description of specific embodiments is not intended to limit the disclosure to cover all modifications, equivalents and alternatives falling within the spirit and scope of the disclosure as defined by the appended claims.

DESCRIPTION OF EXEMPLARY EMBODIMENTS

[0064] The details of one or more embodiments of the presently-disclosed subject matter are set forth in this document. Modifications to embodiments described in this document, and other embodiments, will be evident to those of ordinary skill in the art after a study of the information provided in this document. The information provided in this document, and particularly the specific details of the described exemplary embodiments, is provided primarily for clearness of understanding and no unnecessary limitations are to be understood therefrom. In case of conflict, the specification of this document, including definitions, will control.

[0065] While the terms used herein are believed to be well understood by those of ordinary skill in the art, certain definitions are set forth to facilitate explanation of the presently-disclosed subject matter.

[0066] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as is commonly understood by one of skill in the art to which the invention(s) belong.

[0067] All patents, patent applications, published applications and publications, GenBank sequences, databases, websites and other published materials referred to throughout the entire disclosure herein, unless noted otherwise, are incorporated by reference in their entirety.

[0068] Where reference is made to a URL or other such identifier or address, it is understood that such identifiers can change and particular information on the internet can come and go, but equivalent information can be found by searching the internet. Reference thereto evidences the availability and public dissemination of such information.

[0069] As used herein, the abbreviations for any protective groups, amino acids and other compounds, are, unless indicated otherwise, in accord with their common usage, recognized abbreviations, or the IUPAC-IUB Commission on Biochemical Nomenclature (see, Biochem. (1972) 11(9):1726-1732).

[0070] Although any methods, devices, and materials similar or equivalent to those described herein can be used in the practice or testing of the presently-disclosed subject matter, representative methods, devices, and materials are described herein.

[0071] Following long-standing patent law convention, the terms “a”, “an”, and “the” refer to “one or more” when used in this application, including the claims. Thus, for example, reference to “a biomarker” includes a plurality of such biomarkers, and so forth.

[0072] Unless otherwise indicated, all numbers expressing quantities of ingredients, properties such as reaction conditions, and so forth used in the specification and claims are to be understood as being modified in all instances by the term “about”. Accordingly, unless indicated to the contrary, the numerical parameters set forth in this specification and claims are approximations that can vary depending upon the desired properties sought to be obtained by the presently-disclosed subject matter.

[0073] As used herein, the term “about,” when referring to a value or to an amount of mass, weight, time, volume, width, length, height, concentration or percentage is meant to

encompass variations of in some embodiments $\pm 10\%$, in some embodiments $\pm 5\%$, in some embodiments $\pm 1\%$, in some embodiments $\pm 0.5\%$, and in some embodiments $\pm 0.1\%$ from the specified amount, as such variations are appropriate to perform the disclosed method.

[0074] As used herein, ranges can be expressed as from “about” one particular value, and/or to “about” another particular value. It is also understood that there are a number of values disclosed herein, and that each value is also herein disclosed as “about” that particular value in addition to the value itself. For example, if the value “10” is disclosed, then “about 10” is also disclosed. It is also understood that each unit between two particular units are also disclosed. For example, if 10 and 15 are disclosed, then 11, 12, 13, and 14 are also disclosed.

[0075] As used herein, “optional” or “optionally” means that the subsequently described event or circumstance does or does not occur and that the description includes instances where said event or circumstance occurs and instances where it does not. For example, an optionally variant portion means that the portion is variant or non-variant.

[0076] As used herein, the term “subject” refers to a target of administration. The subject of the herein disclosed methods can be a mammal. Thus, the subject of the herein disclosed methods can be a human, non-human primate, horse, pig, rabbit, dog, sheep, goat, cow, cat, guinea pig or rodent. The term does not denote a particular age or sex. Thus, adult and newborn subjects, as well as fetuses, whether male or female, are intended to be covered. A “patient” refers to a subject afflicted with a disease or disorder. The term “patient” includes human and veterinary subjects.

[0077] As used herein, the terms “administering” and “administration” refer to any method of providing a pharmaceutical preparation to a subject. Such methods are well known to those skilled in the art and include, but are not limited to, oral administration, transdermal administration, administration by inhalation, nasal administration, topical administration, intravaginal administration, ophthalmic administration, intraaural administration, intracerebral administration, rectal administration, and parenteral administration, including injectable such as intravenous administration, intra-arterial administration, intramuscular administration, and subcutaneous administration. Administration can be continuous or intermittent. In various aspects, a preparation can be administered therapeutically; that is, administered to treat an existing disease or condition. In

further various aspects, a preparation can be administered prophylactically; that is, administered for prevention of a disease or condition.

[0078] As used herein, the term “effective amount” refers to an amount that is sufficient to achieve the desired result or to have an effect on an undesired condition. For example, a “therapeutically effective amount” refers to an amount that is sufficient to achieve the desired therapeutic result or to have an effect on undesired symptoms, but is generally insufficient to cause adverse side effects. The specific therapeutically effective dose level for any particular patient will depend upon a variety of factors including the disorder being treated and the severity of the disorder; the specific composition employed; the age, body weight, general health, sex and diet of the patient; the time of administration; the route of administration; the rate of excretion of the specific compound employed; the duration of the treatment; drugs used in combination or coincidental with the specific compound employed and like factors well known in the medical arts. For example, it is well within the skill of the art to start doses of a compound at levels lower than those required to achieve the desired therapeutic effect and to gradually increase the dosage until the desired effect is achieved. If desired, the effective daily dose can be divided into multiple doses for purposes of administration. Consequently, single dose compositions can contain such amounts or submultiples thereof to make up the daily dose. The dosage can be adjusted by the individual physician in the event of any contraindications. Dosage can vary, and can be administered in one or more dose administrations daily, for one or several days. Guidance can be found in the literature for appropriate dosages for given classes of pharmaceutical products. In further various aspects, a preparation can be administered in a “prophylactically effective amount”; that is, an amount effective for prevention of a disease or condition.

[0079] As used herein, the term genetic manipulation includes methods that result in changes to a patient’s protein levels or protein activity at the genetic level which includes but is not limited to: genetic knock-down, genetic knockout, genetic mutation, genetic silencing by miRNA, siRNA, or shRNA, and CRISPR derived base editor technology.

[0080] As used herein, the term “pharmacological intervention” includes but is not limited to administering to a patient small molecules, natural products, compounds, pharmaceutical mixtures and pharmacological agents in a pharmaceutically acceptable carrier. For example, it is commonly understood in the art that insulin, metformin, sulfonylureas, meglitinides, GLP-1 receptor agonists, DPP-4 inhibitors, and SGLT2 inhibitors are all pharmacological interventions for the treatment of diabetes.

[0081] As used herein the term biological intervention includes but is not limited to: administering to a patient antibodies, antibody fragments, stem cells, proteins, peptides, viral carriers, various method of genetic manipulation, and biologic agents in a pharmaceutically acceptable carrier.

[0082] As used herein the term “lifestyle changes” includes but is not limited to dietary changes, exercise regimens, or the implementation of nutritional programs commonly recognized in the art to serve as therapy for metabolic diseases which include pre-diabetes and diabetes.

[0083] As used herein, the terms “comorbidities of type-2 diabetes” or “type-2 diabetes and comorbidities thereof” includes certain diseases well recognized in the art in which patients with type-2 diabetes are known to be at increased risk of developing. These common comorbidities include such diseases as obesity, heart failure, cancer, stroke and the like. Comorbidities may also be referred to, in the art, as diabetic complications.

[0084] As used herein, inhibitors of Soluble epoxide hydrolase can include reversible and irreversible inhibitors, full or partial inhibitors that directly or indirectly bind to soluble epoxide hydrolase. It is well known in the art that UC1153, GSK2256294, and SMTP-7 are soluble epoxide hydrolase inhibitors.

[0085] As used herein, an anti-diabetic therapeutic includes common treatments known in the art including but not limited to: insulin, metformin, sulfonylureas, meglitinides, GLP-1 receptor agonists, DPP-4 inhibitors, SGLT2 inhibitors and combinations thereof.

[0086] As used herein, anti-cancer therapeutics include but are not limited to the following general classes of therapeutics as is well known in the art: alkylating agents, anthracyclines, cytoskeletal disruptors, epothilones, histone deacetylase inhibitors, topoisomerase I and II inhibitors, kinase inhibitors, nucleoside analogues, peptide antibiotics, platinum agents, retinoids, vinca alkaloids, derivatives thereof, and combinations thereof.

[0087] As used herein, heart failure therapeutics include but are not limited to: Aldosterone antagonists, ACE inhibitors, ARBs (angiotensin II receptor blockers), ARNIs (angiotensin receptor-neprilysin inhibitors), Beta-blockers, Blood vessel dilators, Calcium channel blockers (unless systolic heart failure), Digoxin, Diuretics, Heart pump medication, Potassium or magnesium, Selective sinus node inhibitors, and combinations thereof.

[0088] As used herein, a therapeutic for the treatment of stroke, including but not limited to ischemic stroke, includes: anti-coagulants, anti-platelet therapeutics, tissue plasminogen

activator, statins, angiotensin-converting enzyme (ACE) inhibitors, beta-blockers, calcium channel blockers, and combinations thereof.

[0089] The presently-disclosed subject matter is further illustrated by the following specific but non-limiting examples. The following examples may include compilations of data that are representative of data gathered at various times during the course of development and experimentation related to the present invention.

[0090] The details of one or more embodiments of the presently-disclosed subject matter are set forth in this document. Modifications to embodiments described in this document, and other embodiments, will be evident to those of ordinary skill in the art after a study of the information provided in this document. The information provided in this document, and particularly the specific details of the described exemplary embodiments, is provided primarily for clearness of understanding and no unnecessary limitations are to be understood therefrom. In case of conflict, the specification of this document, including definitions, will control.

[0091] The presently-disclosed subject matter is further illustrated by the following specific but non-limiting examples. The following examples may include prophetic examples, notwithstanding the numerical values, results and/or data referred to and contained in the examples. The following examples may include compilations of data that are representative of data gathered at various times during the course of development and experimentation related to the present invention.

EXAMPLES

[0092] Methods

[0093] Animal Models. The HIP rat is a Sprague-Dawley (SD) rat that expresses human amylin in pancreatic β -cells on the insulin II promoter. HIP rats have a ~3-fold increase of amylin secretion³⁸, similar to humans with hyperamylinemia^{29,30}. HIP rats have amylin deposition in pancreas³⁸ and peripheral organs^{6,16,71}, including the heart⁶. Quantitative real-time PCR showed no presence of human amylin mRNA in the HIP rat heart⁷, indicating that amylin deposited in the heart comes from the circulation, as observed in humans^{6,7}. In the present invention HIP rats with eight backcrosses to SD rats are used. Amylin oligomerization and deposition in the pancreas leads to a gradual decline of β -cell mass, glucose dysregulation by ~7-9 mo of age¹⁶ and T2D by 10-12 mo of age¹⁶. Insulin and amylin secretion are maximum at ~8 mo of age, followed by a decline with the development

of T2D¹⁶. Symptoms in terminal stages include lethargy and neurological deficits¹⁸. Sudden death and arrhythmia²⁵ are more frequent in male HIP vs. WT littermate rats. Female HIP rats show overt hyperglycemia later in life (i.e., 18 mo of age), as previously reported¹⁸. Physical deterioration is milder in females compared to male HIP rats¹⁸. AKO mice have been used previously¹⁰ to investigate the interaction of exogenous amylin (aggregated vs. monomeric) with the sarcolemma. To circumvent species-specific paradigms in comparing mice vs. rats, the amylin knockout (AKO) rats, were generated (see Ref 18, for details).

[0094] Diet interventions. High-fat diet (60% kcal as fat; Research Diets, Inc) or chow *ad libitum* are used to discriminate effects of hyperamylinemia from known cellular and metabolic disturbances in diabetic heart.

[0095] RBC amylin: Amylin levels in RBCs from HIP and WT rats is measured (both males and females) in longitudinal studies. Flow cytometry (Becton Dickinson LSRII; as in **Fig 4A**) is used to measure amylin deposition on RBCs, followed by ELISA, Western blot and mass spectroscopy of amylin in RBC lysates, as described previously^{7,8,16-18}. The inventors developed a specialized ELISA⁷⁴ and can measure amylin levels in up to 400 RBC samples in parallel, which is ideal for longitudinal studies. Because diabetes develops later (by 6 mo) in female vs. male HIP rats, it is anticipated that the RBC amylin level will increase in males, but not significantly change in female HIP rats (until 16-18 mo. of age), suggesting an estrogen effect.

[0096] Amylin uptake. To investigate effects on RBC amylin uptake induced by changes in the metabolic milieu, high-fat diet in HIP vs. WT rats were tested. Rats of 6 mo. of age (normal blood glucose levels in HIP rats) were fed insulinogenic or chow diet *ad libitum* for 6 mo. and monitored as described herein. Characteristic histograms for RBC amylin levels are derived both at baseline and with longitudinal data.

[0097] RBC deformability: In all rat groups ($\pm$ high-fat diet), the RBC shape distributions are analyzed by FACScan flow cytometry (Becton Dickinson LSRII) as a function of characteristic RBC amylin levels, as in **Fig 4D**. The Pearson coefficient of dissymmetry (PCD) shown in **Fig 4D** reflects the sphericity of RBCs (PCD=0 indicates RBC spherical shape). The data (**Fig 4D**) suggest that amylin deposition on the RBC surface (or incorporation of amylin inside the cell) negatively affects the RBC deformability.

[0098] RBC Hb and HbO₂ levels: RBC specimens from each rat group will be suspended at a hematocrit of 2.5% in HBSS/A, followed by the incubation with 0.5% sodium dodecyl

sulfate (SDS) for 5 hours. The total amount of Hb in 100 μ L RBCs suspension is calculated from the optical density measured by a spectrophotometer (absorption peak at 410 nm, as in **Fig 4E**). Hb level measured in **Fig 4E** is the sum of oxygenated Hb (HbO₂) plus deoxygenated Hb mass. HbO₂ and Hb have different absorption spectra (maxima at 410 nm and 430 nm, respectively), which is used to estimate amylin induced changes in HbO₂ and Hb RBC content. All RBC samples are tested for Hb levels using high-performance liquid chromatography (HPLC), as described previously⁸.

[0099] Adherence of amylin-loaded RBCs to capillary endothelium: RBCs collected from each HIP rat group (normal, pre-diabetes, diabetes, $\pm$ high-fat diet) are infused into AKO rats (300 μ L RBCs, daily, 7 days, q.d., via the tail vein). Because diabetes develops later (by 6 mo) in female vs. male HIP rats, AKO rats are matched for age and sex to HIP rats providing the RBCs for infusion. At the end of infusion period, rats are sacrificed, perfused with saline through a catheter implanted into the right external jugular vein to wash out the blood from blood vessels, followed by heart collection and paraffin embedding. Confocal microscopy is used to identify the attachment of amylin-loaded RBCs to vascular endothelium (as in **Fig 5A**). Before analysis, heart sections are incubated with 1% Sudan black to block autofluorescence. Control sections with either omitted primary or secondary antibodies are used to establish specific staining. The co-localization of amylin, glycophorin A and collagen IV (as in **Fig 5B**) signifies HIP rat RBCs attached to the vascular endothelium. To further investigate the relationship between RBC amylin level and RBC stickiness, ex vivo experiments are performed. Cardiac microvascular endothelial cells are used from WT adult SD rats (RA-6024, Cell Biologics, IL). The cells are plated on 96-well plate coated with cell attachment factor solution (123-100, Sigma, MO), allowed to attach for 24 hours and used for experiment when they reached 70%-90% confluency. HIP rat RBCs collected from the same groups as in above are gently layered on confluent endothelial cells and incubated at 37°C for 40 minutes. The plate is then inverted and allowed to sit for an additional 30 minutes at 37°C. With the plate maintained in the inverted position, the adhesive sheet will be removed followed by the measurement of the Hb content (as described below) from the optical density (with a spectrophotometer). Adherence is calculated as percentage of optical density of adhered Hb (as in **Fig 5C**).

[00100] Amylin deposition in capillaries: Immunohistochemistry methods are used to identify amylin deposition in cardiac blood vessels and confocal microscopy to identify the anatomical localization of amylin, as before^{6,7}.

[00101] Hypoxia: As the endpoint, plasma EPO levels (by ELISA; as in **Fig 6**) and cardiac hypoxia markers in all rat groups are measured. Under hypoxia, HIF-1 α is stabilized and moves into the nucleus, where it regulates transcription of genes whose protein products increase oxygen delivery or facilitate metabolic adaptation to hypoxia⁷⁵⁻⁷⁷. The VHL/HIF oxygen-sensing pathway and HIF-1 markers⁷⁵⁻⁷⁷ in cardiac tissue, including VEGF-A, PGK1 and NOS, using qRT-PCR for expression and ELISA for protein levels is also investigated. Longer term response to hypoxia (which may be the case of diabetic HIP rats) is HIF-2 α dominated⁷⁷. HIF-2 α regulates EPO⁷³ (which is upregulated in HIP rats; **Fig 6**) and Arginase (Arg)⁷⁸. Arg1 & Arg 2 is measured (using qRT-PCR for expression and ELISA for protein levels; as in **Figs 8A,B**), Arg activity (using Arginase assay kit; as in **Figs 8C**) and NO availability (by ELISA) in cardiac tissues from all rat groups.

[00102] Structural integrity and stability of myocardial capillaries. HIP and WT rats and AKO rats infused with either aggregated amylin or monomeric (functional) amylin was investigated. Anatomical distribution of aggregated amylin (capillary vs. myocardial interstices) is assessed by immunohistochemistry, as previously described⁶⁻⁸. The co-localization of amylin deposits, inflammation and microhemorrhages by serial staining of myocardial tissue with amylin antibody, inflammatory markers (IBA-1 and ED1) and Prussian blue (as in **Fig 9 & 10**) was tested in all rat groups. The distribution of aggregated amylin relative to myocardial capillaries and sarcolemma is assessed by using cryo-immunogold labeling. For this, freshly isolated heart samples are incubated overnight in 1.85M sucrose/20% PVP-10/50mM Hepes pH 7.4. Tissue cuts will be mounted on an aluminum pin and quick frozen in liquid nitrogen. Ultrathin sections are collected on nickel grids with plastic/carbon film and stained with antibodies. The secondary antibodies are conjugated to 5, 10 or 12 nm gold particles. Imaging is done at the EM facility at UK using a transmission EM (JEM-1230; JEOL, Japan) operated at 120 keV, as in previously reported⁷.

[00103] Endothelial basement membrane structure (immunostaining for collagen IV, as in **Fig 10A**) and mural cell coverage of myocardial capillaries (immunostaining for CD31, caveolin and ulex europaeus, as in **Fig 10C**) is investigated in groups. Altered permeability of myocardial capillaries is assessed by comparing the expression (via qRT-PCR) and protein levels (Western blot, immunoprecipitation and/or ELISA) of components of adherens junctions (vascular endothelial cadherin; Cdh5, and α -actinin; Actn) and tight junctions (claudins, occludin and ZO) in isolated myocardial capillaries from HIP vs. WT rats (as in **Fig 9D**). For isolation of capillaries, myocardial tissues in liquid nitrogen are snap frozen,

minced into small pieces in ice cold cPBS and then homogenized in ice cold cPBS using glass homogenizer. The homogenates are added into ficoll solution (30%). Samples are mixed by gently inverting tubes and then centrifuged (at 5800g for 20 minutes at 4°C). Supernatants are discarded and the pellets are re-suspended in ice cold cPBS with 1%BSA. Suspensions are passed through 300 micron nylon mesh strainer. Glass beads and pellet suspension are added on the top of column, allowed for 1-2 min to settle and passed through column with the rate of one drop per second. Beads are inverted into 100mL beaker and 20mL cPBS with 1%PBS and then centrifuged (500g for 5 minutes at 4°C). After centrifugation pellets are re-suspended in ice cold cPBS. Capillaries are stained with Texas Red dye and quality is assessed under confocal microscope. (**Fig 9D**).

[00104] Sarcolemmal processes and myocyte viability: Cardiac myocytes were isolated from rats in all rat groups. Isolated myocytes are used for testing myocyte accumulation of amylin (by ELISA and Western blot, as shown before^{8,10}), sarcolemmal structural integrity (dye leakage assay, electron microscopy, LC-MS/MS and fluorescent markers) and myocyte Ca²⁺ cycling (see below). Each experiment is performed on hearts/myocytes from a number of rats/group. Calculation of the minimum sample size with a two-tail t-test considering $\alpha=0.05$ and $1-\beta=0.8$; performed with GPower 3.1.9.2.

[00105] Dye leakage assays are performed to test the effect of aggregated amylin on sarcolemmal integrity. The uptake of the fluorescent dyes Lucifer Yellow (MW=443 Da), Alexa-488 (MW= 570 Da) and calcein (MW=623 Da) in cardiac myocytes using a scanning confocal microscope to determine whether the pores created by aggregated amylin are large enough to allow the passage of large molecules is measured.

[00106] Cryo-EM is used for *ultrastructural analysis* of cardiac myocytes, as before^{7,83}. Briefly, myocytes are frozen rapidly and embedded in Eponate-12 resin. Blocks are cut into 70-nm-thick sections. Serial sections are collected on slot grids covered with a 50 nm Formvar support film and visualized on the EM.

[00107] Lipid peroxidation is measured as previously described⁸. Sarcolemmal lipid peroxidation is measured using confocal microscope analysis of the fluorescent probe 4,4-difluoro-3a,4adiaza-s-indacene (C11-BODIPY^{581/591}, Invitrogen) in isolated cardiac myocytes. This probe is efficient for assessing the free radical-mediated oxidation that takes place in the lipophilic domain, while it does not detect aqueous radicals⁸⁸. Formation of amylin-4HNE and amylin-MDA adducts in cardiac tissue is also measured using LC-MS/MS and Western

blot, as before⁸. Myocyte ROS levels in HIP vs. WT rats are measured using fluorescent indicators CM-H₂DCFA (H₂O₂), as described previously^{7,8}.

[00108] EET effect on myocytes. Pre-diabetic HIP rats that have lower RBC amylin and RBC deformability levels are used. Selected HIP rats will presumably have intact vascular endothelial cells. To increase their endogenous levels of EETs, HIP rats are treated with 1ml drug/liter APAU sEH inhibitor (UC1153, from *abcam*) in drinking water for 10 weeks, as previously described^{7,83}. Rats in each of the four EET treatment groups are intravenously infused with 10 mg/Kg EETs via tail vein, weekly, for 10 weeks. Control rats (matched for age and blood glucose, RBC amylin and RBC deformability levels) received the same amount of polyethylene glycol, a neutral biocompatible polymer (vehicle). Blood glucose and RBC characteristics (amylin, deformability and stickiness) are measured biweekly. At the end of treatment period, heart function is measured, followed by euthanasia and measurement of cardiac hypoxiamarkers, structural integrity and stability of capillaries and sarcolemmal processes.

[00109] Sub-sarcolemmal deposits. To clarify whether aggregated amylin accumulates intracellularly, LC-MS/MS analysis of sarcolemmal vs. cytosolic amylin fractions was performed. To separate the sarcolemmal fraction, myocyte lysates are centrifuged at 1,000 g for 5 min to generate a postnuclear supernatant. This is then further centrifuged at 27,000 g for 35 min and the plasma membrane pellet is re-suspended in Laemmli buffer. This protocol results in efficient separation of sarcolemmal (SL) and soluble fractions (Cyt) (**Fig 12**), i.e. the sarcolemmal protein Caveolin3 is found almost exclusively in the SL fraction while the cytosolic protein GAPDH is highly enriched in the soluble fraction. Specimens from each group are further analyzed by confocal microscopy to identify anatomical localization of amylin deposits. Sections are treated with formic acid to improve immunostaining. Serial sections 50 µm thick are incubated in biotinylated amylin antibody overnight at 25°C. For confocal microscopy, biotinylated amylin antibody is visualized using streptavidin conjugated Cy-3 fluorochrome. Confocal images are collected on an Olympus IX70 inverted microscope. For blocking tissue autofluorescence, slides are pre-incubated in 1% Sudan black solution for 1h at room temperature. Control sections with either the primary or secondary antibodies omitted are used to test the antibody specificity.

[00110] Since myocyte Ca²⁺ dysregulation in diabetes involves RyR activation by post-translational modifications^{81,82}, RyR phosphorylation & O-GlcNAcylation using

immunoblots and phospho-specific antibodies²⁵ is assessed while RyR oxidation is assessed with the monobromobimane method⁸⁷ as in **Fig 13**.

[00111] Glycated amylin is prone to faster aggregation⁹⁵, which is also suggested the data (**Fig 14**). Hyperglycemia and hyperamylinemia together could further enhance accumulation of aggregated amylin in RBCs and the cardiovascular system. LC/MS/MS and Pro-Q Emerald glycoprotein gel stain kit (Molecular Probes) is used to measure glycated amylin and test whether hyperglycemia increases amylin aggregation, *in vivo*.

[00112] Results

[00113] Aggregated amylin in circulating RBCs in humans. Both monomeric amylin (3.9 kDa) and aggregated amylin are present in the blood of humans with obesity and HF, as previously reported⁸. Matched plasma, RBCs and white blood cells (WBCs) from the same patient showed multiple molecular weight bands positive for amylin (**Fig 2A**) consistent with previous data⁷ from HIP rats demonstrating that amylin forms detergent-insoluble amylin aggregates in the blood. The large molecular weight bands in **Fig 2A** may also signify interactions of amylin with other blood proteins, lipids or circulating reactive aldehydes, as suggested by recent studies¹⁸ of plasma from HIP rats. The amylin immunoreactivity signal in RBCs is greatly increased compared to plasma and WBCs. Paired RBCs and cardiac myocytes from the same patients have almost identical size distribution of incorporated amylin (**Fig 2B**), suggesting a mechanism of cardiac amylin accumulation based on pre-formed aggregated amylin secreted in the blood from pancreatic islets. A study was performed to determine a possible association between diabetic HF and elevated RBC amylin levels. n=110 RBC specimens and medical record data stored at the University of Kentucky Center for Clinical and Translational Science (UK-CCTS) were tested. Patients in the HF group suffered with idiopathic dilated cardiomyopathy. RBC samples were provided by individuals with obesity (BMI~30) and HF (O-HF; n=17), obesity without HF (O-NF; n=22), no obesity (BMI<30) with HF (Non-O-HF; n=23) and no obesity, no HF (controls; Non-O; n=48). The results (**Fig 2D**) indicate that HF correlated with increased levels of amylin in RBCs. Compared to BMI<30, a BMI~30 appears also to increase the RBC amylin level.

[00114] Western blot on pancreas homogenates demonstrates lack of amylin immunoreactivity signal in AKO rats (**Fig 3A**). Deletion of amylin gene reduced the difference in the blood glucose elimination between male and female rats (**Fig 3B**), consistent with data⁸⁶ reported in amylin knockout mice. Intriguingly, the increased insulin responses

are associated with weight gain in male AKO vs. WT rats, whereas females appear unaffected (**Fig 3C**).

[00115] Circulating RBCs accumulate aggregated amylin. The RBC amylin level in HIP rats increased with the pathological progression from normal to pre-diabetic and to diabetic states, as shown by flow cytometry data (**Fig 4A**), ELISA (**Fig 4B**) and Western blot (**Fig 4C**). Compared to WT rats, which have RBCs with normal biconcave disc shapes, the RBCs from HIP rats have rounded shapes (**Fig 4D**) and decreased hemoglobin (Hb) levels (**Fig 4E**). This structural change of RBCs was replicated by the incubation of WT rat RBCs with recombinant human amylin (50 $\mu\text{mol/L}$; 2 h; **Fig 4F**).

[00116] Aggregated amylin changes the interaction of RBCs with vascular endothelium. as suggested by data (**Fig 5**) from WT rats infused with RBCs collected from diabetic HIP rats (300 μL , daily, for seven days, q.d., via tail vein). Confocal microscopy analysis showed areas of occluded capillaries, indicating the attachment of HIP rat RBCs to endothelium (**Fig 5A**). In contrast, cardiac tissue from WT rats infused with WT rat RBCs showed intact myocardial capillaries. Immuno-staining cardiac tissues for amylin (green), collagen IV (red) and glycophorin A, a RBC specific protein (blue) showed that amylin and glycophorin A are co-localized with collagen IV (**Fig 5B**, overlay), indicating that amylin-loaded RBCs stuck to the vascular wall. Consistent with these results, RBCs from HIP rats have increased adherence to cultured endothelial cells (**Fig 5C**). Incubation of RBCs from WT rats with recombinant human amylin (50 $\mu\text{mol/L}$; 2 h) also increased the RBC adherence to cultured endothelial cells (**Fig 5C**).

[00117] The plasma levels of erythropoietin (EPO), a glycoprotein cytokine secreted by kidneys in response to cellular hypoxia⁷³, are greatly increased in HIP rats showing high RBC amylin levels (~ 16 mo old), compared to age-matched WT rats (**Fig 6**). In contrast, HIP rats in the normal state (6 mo old) and age-matched WT rats have similar plasma EPO levels (**Fig 6**).

[00118] Amylin deposition in capillaries and RBCs damages capillary endothelium, enabling infiltration of aggregated amylin into myocardial interstices and injury of myocyte sarcolemma. Myocardial tissues from HIP rats showed abundant haemosiderin deposits (**Fig 9A**) indicating microhemorrhages, which is consistent with previous findings in HIP rat brains. Infusion of RBCs from HIP rats into normal WT rats provoked sporadic myocardial microhemorrhages (**Fig 9B**). In contrast, WT rats infused with WT rat RBCs showed no

microhemorrhages (**Fig 9C**). In HIP rats, microhemorrhages were found in association with mild edema (**Fig 9A**; arrow), suggesting altered tight junctions. Indeed, the levels of tight junction proteins (claudin, occludin and ZO-1) were lower in myocardial capillaries from HIP rats compared to WT littermates (**Fig 9D**), which correlated with amylin accumulation in capillaries (**Fig 9E**).

[00119] Co-staining for amylin and collagen IV, a component of the basement membrane structure, showed that amylin (brown) is co-localized with collagen (green) in small blood vessels (presumable capillaries and arterioles; **Fig 10A**). Collagen was replaced with amylin deposits in capillary beds, which correlated with dilation of capillaries, increased separation of the myocytes and myocyte disarray. Co-staining for amylin and the ionized calcium-binding adapter molecule 1 (IBA1), a marker of macrophage activation, indicated abundant intravascular accumulation of macrophages in HIP rat hearts (**Fig 10B**). Macrophages (green) are seen in areas of amylin deposition (brown) suggesting endothelial injury and a potential role of macrophages in the clearance of aggregated amylin from blood vessels. Myocardial capillaries appeared depleted of mural cell coverage in HIP vs. WT rats (immunostaining for *ulex europaeus*, **Fig 10C**). In contrast, no microhemorrhages, amylin deposition, macrophage accumulation and endothelial cell loss are seen in myocardial tissues from WT littermate rats (**Fig 9 & 10**).

[00120] Amylin levels in plasma, RBCs and myocardial capillary lysates from WT rats infused with HIP rat RBCs are elevated compared to WT rats infused with WT rat RBCs (**Fig 11A**). The presence of amylin deposition in myocardial microvessels from WT rats infused with HIP rat RBCs was confirmed by immunohistochemistry with an amylin antibody (brown; **Fig 11B**). In cardiac tissues from WT rats infused with HIP rat RBCs, co-staining for amylin and IBA1 indicated intravascular macrophage activation (**Fig 11C**). In contrast, infusion of WT rats with similar amounts of WT rat RBCs showed no effect on blood amylin level and capillary structure.

[00121] The 14,15-EET isomer reduced amylin aggregation (**Fig 15A**) and amylin incorporation in cardiac myocytes (**Fig 15B**) *ex vivo*.

[00122] The use of membrane sealants, such as poloxamer 188 (P188), prevented cardiac injury and ventricular dilation in dystrophic mice⁸⁴ and dogs⁶⁸. A single intravenous P188 bolus significantly reduced membrane damage and improved compound muscle action potential in a rat model of membrane injury, as previously reported⁶⁷. P188 prevents the

increase in sarcolemmal Ca^{2+} leak and the $[\text{Ca}^{2+}]_i$ increase in myocytes incubated with aggregated amylin (**Fig 16**), consistent with previous data reported for $\text{A}\beta$ in neurons⁸⁵. Acute exposure to P188 (2 h) also reduced lipid peroxidation in myocytes isolated from diabetic HIP rats (**Fig 17**).

Discussion

[00123] These data suggest the following: *i*) circulating RBCs take up and concentrate aggregated amylin, *ii*) amylin-loaded RBCs have lower Hb levels, *iii*) the RBC amylin level increases with the progression of diabetes, and *iv*) compared to normal RBCs, amylin-loaded RBCs have anomalous stiffness and stickiness, jamming the capillaries. Thus, elevated blood levels of aggregated amylin and subsequent amylin deposition in RBCs may provoke cardiac hypoxia by impairing RBC deformability, reducing the Hb content in RBCs and slowing the RBC move through the capillaries.

[00124] ADDITIONAL or ALTERNATIVE MATERIALS & METHODS

[00125] Human Studies

[00126] In some embodiments, this research employed de-identified blood specimens matched with medical record data obtained from the biobank of the Center for Clinical and Translational Science at University of Kentucky (UK-CCTS). Sample collection and storage was approved by the Institutional Review Board at the University of Kentucky. Written informed consent was received from each individual prior to donating the blood sample. Specimens from transplant recipients (less than 6 months from the transplant), patients with

[00127] liver disease, patients with HIV and pregnant or lactating women, which may affect the pancreatic secretion of amylin, were excluded from the study. RBCs from patients with type-2 diabetes were the amylin positive control, whereas patients with over 15 years of type-1 diabetes (and, therefore, depleted β -cell mass) but otherwise healthy served as the negative control for amylin. The latter samples were collected under a different IRB-approved protocol and de-identified.

[00128]	Sample size	[00129]	n = 353		
[00130]		[00131]	Disease (n=287)	[00132]	Healthy (n=66)
[00135]	Age	[00136]	55±1 years	[00137]	43±2 years
[00138]	Female sex	[00139]	127(45%)	[00140]	29(45%)
[00141]	Obesity	[00142]	116(41%)	[00143]	14(21%)
[00144]	Heart Failure	[00145]	108(38%)	[00146]	
[00147]	Type-2 Diabetes	[00148]	69(24%)	[00149]	
[00150]	Cancer	[00151]	91(32%)	[00152]	
[00153]	Stroke	[00154]	13(5%)	[00155]	
[00156]	Type-1 Diabetes	[00157]	5(2%)	[00158]	

Table 2. Characteristics of the individuals providing the RBC samples, including age, sex, body mass index (BMI), diabetes status and co-morbidities.

[00159] RBC specimens were divided into groups based on the primary diagnosis of heart failure, cancer or stroke. Most individuals in the disease group had type-2 diabetes as the second diagnosis. Diabetes status, co-morbidities and characteristics of the individuals providing the blood samples, such as age, sex and body mass index (BMI) are summarized in Table 2. The control group represents individuals without diabetes, heart failure, stroke or cancer.

[00160] Statistics and defining and handling of outliers for the human study

[00161] For some analyses, the homogeneity of variance was tested using Levene's test. Equal variance was assumed when $P > 0.05$ and normal t-test (parametric test) was applied. When ANOVA assumptions were violated, Mann-Whitney test (non-parametric test) was applied. Correlation estimates with scatterplots were used to examine the relationship between RBC amylin levels and HbA1c (Figs. 18 d-j), respectively, in healthy individuals and disease groups. All analyses were performed using GraphPad Prism 5.0 software.

[00162] A Whisker plot was computed (Fig. 18c) from amylin levels in human RBC across different groups. In each group, the median, first and third quartiles were calculated. The interquartile range was calculated by subtracting the lower quartile value from the upper quartile value. Points falling more than 1.5 times the interquartile range above the third quartile were considered outliers. The outliers were indicated in the Fig. 18 caption and included in statistical analyses.

[00163] Experimental Animals

[00164] All animal experiments were approved by the Institutional Animal Care and Use Committee at University of Kentucky and conforms to the Guide for the Care and Use of Laboratory Animals, the 8th Edition, published by the National Academies Press, Washington (DC); 2011. Rats that develop type-2 diabetes (T2D) linked to expression of human amylin in the pancreas (HIP rats; n=62) were compared with rats that develop T2D in the absence of amyloid, as they express solely the non-amyloid forming rat amylin (UCD rats; n=37) and control, non-diabetic rats (WT rats; n=50). Only males were used because HIP females become diabetic at a more advanced age. (FIGs. 24-25)

[00165] HIP rats are Sprague-Dawley (SD) rats that overexpress (3-fold) human amylin specifically in the pancreatic f3-cells on the insulin II promoter. (FIG. 24) HIP rats develop amylin dyshomeostasis and amylin amyloid deposition in pancreatic islets leading to f3-cell apoptosis and hyperglycemia with regular diet. Breeding pairs were obtained from Charles River Laboratory. The presence of the human amylin gene was confirmed by standard PCR techniques to detect Rip1 and Mus-a-actin. Actin was used as the internal control. WT littermates were used as the non-diabetic control.

[00166] UCD rats were generated as described in Ref 106 by crossing obese Sprague-Dawley rats with Zucker diabetic fatty (ZDF) lean rats. ZDF-lean founder rats were purchased from Charles River Laboratories. These rats have functional leptin receptors and the autosomal recessive f3-cell defect. (FIG. 26) The breeding strategy was to maintain the obese phenotype from obese Sprague-Dawley rats and selecting for individuals homozygous for the autosomal recessive f3-cell defect. In the seventh generation (F7) all animals were homozygous for the f3-cell defect and increased genetic propensity to develop diabetes.

[00167] Rats were housed (singly) in individually ventilated cages (ACE, Allentown, NJ) with Sani-Chip bedding (Harlan-Teklad) and maintained on a standard chow diet (Teklad Global 18% Protein Rodent Diet #2018) and reverse osmosis drinking water for the duration of the study. Nesting material (Enviro-Dri®, Shepherd Specialty Papers) and aspen chew blocks (Lomir Biomedical Inc.) were provided for environmental enrichment. All animals were maintained on a 12:12 hour light:dark cycle at temperatures between 21-24 °C.

[00168] Treatment

[00169] A retrospective analysis was performed of kidney tissue from HIP rats with pharmacologically elevated EETs used in a prior study. (FIG. 27). Briefly, pre-diabetic male

HIP rats were randomized to treatment (HIP-T) and no treatment (HIP-UT) groups by a blinded observer. To increase the endogenous levels of EETs, pre-diabetic HIP rats were treated with 1 ml drug/L of the soluble epoxide hydrolase inhibitor APAU (UC1153) added in drinking water for 10 weeks. The treatment resulted in lower levels of aggregated amylin in blood and cardiac myocytes and improved heart function.

[00170] Renal function analysis

[00171] Rats were placed in metabolic cages. After 2 days of acclimation, 24-hours cycle urine collections were taken at baseline and day 2. Blood sampling (500 µl) was obtained from tail vein for determination of creatinine level. Creatinine concentration in plasma and urine was measured using the kinetic Jaffé method (FIG.s 28A-C) with 10% picric acid solution in NaOH. After 15 min, absorbance reading was obtained in a microplate set for dual wavelengths at 490 nm (read) and 620 nm (reference). Creatinine clearance (Ccr) was calculated using the following formula: $Ccr\ (ml/min) = (urine\ creatinine / plasma\ creatinine) \times urine\ flow\ rate$. Urinary excretion rate of albumin was measured using a commercially available ELISA kit (E110-125, Bethil Laboratories, Inc, TX).

[00172] Tissue extraction and RBC isolation

[00173] For measurements of hypoxia markers (HIF-1 α , HIF-2 α , Arginase 1, Arginase 2 and vHL) by ELISA and arginase activity assay, tissues were extracted in 1xPBS with 1% (v/v) protease and phosphatase inhibitors. The homogenate was frozen at -80°C and thawed on ice for 3 consecutive cycles to break cell membranes. For other biochemical assays, tissues were extracted in homogenization buffer (150 mM NaCl, 50 mM Tris, 50 mM NaF, 2% Triton X-100, 0.1% SDS and 1% (v/v) protease and phosphatase inhibitors). All homogenates were then centrifuged at 17,000 xg for 30 minutes at 4°C.

[00174] In some embodiments, rat blood samples were collected into sterile K2-EDTA vacutainer tubes. RBCs were isolated from plasma by centrifugation at 1,000 xg for 10 minutes, at 4°C, immediately after blood collection. To obtain RBCs lysates for biochemical analyses, RBCs were incubated with cell lysate buffer (10% NP-40, 150 mM NaCl, 10 mM Tris, 2 mM EGTA and 50 mM NaF, 1% (v/v) protease and phosphatase inhibitors) for 30 minutes on the rotor at 40°C, followed by centrifugation at 17000 xg for 30 minutes at 4°C. Supernatant was used for experiments.

[00175] RBC transfusion

[00176] In some embodiments, RBCs were isolated into a heparin lithium tube, washed twice with sterile 1xPBS, combined with storage solution (150 mM HCl, 45 mM dextrose, 45.5 mM mannitol, and 2.2 mM adenine) in a 3:1 v/v ratio, transferred to sterile tubes and stored at 4°C in the dark before injection. Rats received 300 µL of pre-warmed (at 37° C) RBCs solution once daily for 7 days via tail vein injection.

[00177] Hematocrit and hemoglobin measurements

[00178] In some embodiments, the ratio of RBCs to total blood volume (hematocrit; Hct) was measured with an i-SATA analyzer (VetScan i-STAT 1 Handheld Analyzer, ABAXIS) using i-STAT CG8+ cartridges (VetScan i-SATA CG8+ cartridge, ABAXIS, Cat# 600-9001-25) according to the manufacturer's protocol.

[00179] In some embodiments, isolated RBCs were first suspended to a hematocrit 2.5% in Hank's Balanced Salt Solution (HBSS) containing 0.5% BSA (HBSS/A). 10 µL of RBCs suspension was further diluted in 990 µL HBSS/A and 100 µL of this RBCs dilution was incubated with 200 µL 0.5% SDS for 5 hours. Total hemoglobin in the 100 µL RBCs diluted suspension was calculated from the optical density (at 405 nm) measured with a spectrophotometer (Molecular Devices, Menlo Park, CA).

[00180] Assessment of RBC shape and amylin deposition on RBCs by flow cytometry

[00181] In some embodiments, RBCs (1×10^7 cells) were aliquoted to assay tubes, washed twice with 2 mL incubation buffer (0.5 % BSA in PBS), and resuspended in 100 µL incubation buffer. RBCs were incubated with an anti-amylin antibody (1:200, T-4157, Bachem-Peninsula Laboratories, CA) for 1 hour on ice, washed twice with PBS by centrifugation at 1000 xg for 1 minute and then incubated with goat anti-rabbit Alexa Fluor® 488 (A11029, Invitrogen, MA) for 30 min on ice. RBCs were then washed twice before re-suspension in 200 µL PBS, and analyzed by flow cytometer (Becton Dickinson LSRII) within 90 min. To assess the cell shape, RBCs were first gated on a forward scatter (FSC)/side scatter (SSC) plot. The R1 events are visualized using a FSC-A/FSC-H dot plot. For detecting amylin deposited on RBCs, the cells were first gated on a forward scatter (FSC)/side scatter (SSC) plot. RBCs were further gated to determine the amylin signal (Alexa 488), using negative control (no antibody) and positive control to set the upper and lower boundaries.

[00182] RBC adhesion assay

[00183] In some embodiments, adult rat microvascular endothelial cells (RA-6024, Cell Biologics, IL) were cultured on 96-wells cell culture plate coated with cell attachment

factor solution (123-100, Sigma, MO). Cells were allowed to attach for 24 hours and used for experiments when they reached 70%-90% confluency. Isolated RBCs were washed twice with cold HBSS and once with HBSS/A. RBCs were suspended to hematocrit 2.5% in HBSS/A. 10 μ L of RBCs suspension was used to measure the hemoglobin content as described above. Another 300 μ L of RBC suspension aliquot (hematocrit 2.5% in HBSS/A) was gently layered on confluent endothelial cells pre-incubated with 150 μ L of media containing different concentrations of ($\pm$)14(15)-EET (50651, Cayman Chemical, MI). The plate was incubated at 37° C for 40 min. An additional 83 μ L of HBSS/A was gently added to create a slight convex meniscus over each well, and the plate was covered with an adhesive plastic film (89087-692, VWR, IL). The plate was then inverted and allowed to sit for an additional 30 min at 37° C. With the plate maintained in the inverted position, the adhesive sheet was removed and the remaining fluid was removed by aspiration. 200 μ L 0.5% SDS was added to each well, followed by 5 hours incubation at room temperature. The amount of hemoglobin in each well was measured from the optical density (OD) as described above. Adherence was calculated as the percentage of OD of adhered hemoglobin/ (OD of total hemoglobin x dilution factor).

[00184] Immunofluorescence

[00185] In some embodiments, isolated RBCs were incubated with primary antibodies against human amylin and Glycophorin A, followed by incubation secondary antibodies, and imaged with a Nikon A1R confocal microscope. For hemoglobin staining, blood smears on glass slide were fixed and incubated with primary antibodies against hemoglobin and human amylin. Smears were then incubated with secondary antibodies, mounted in mounting media and imaged.

[00186] Biochemical assays

[00187] In some embodiments, ELISA assays for human amylin, erythropoietin, HIF1- α , HIF2- α , arginase 1, arginase 2 and vHL were performed according to the manufacturer's protocols. Arginase activity was measured in kidney homogenates using a colorimetric assay. Western blots were performed on plasma, WBC lysates, and RBC lysates from humans using a primary antibody against amylin.

[00188] Immunohistochemistry

[00189] In some embodiments, isolated RBCs were washed 3 times with 1xPBS, blocked with 10% goat serum for 15 minutes at 37°C, incubated with primary antibodies

against human amylin (1:200, T-4157, Bachem-Peninsula Laboratories, CA) and Glycophorin A (1:100, sc-71159, Santa Cruz Biotechnology, TX) for 15 min at 37°C, followed by incubation with anti-mouse IgG and anti-rabbit IgG secondary antibodies for 15 min at 37°C. Cells were hemolyzed in 1% saponin in PBS, centrifuged at 8000g for 5 min and the pellet was re-suspended in 1xPBS for microscopy. Images were obtained on a Nikon A1R confocal microscope (Nikon).

[00190] For hemoglobin staining, blood smears on glass slide were fixed in acetone/ethanol/methanol (6:2:2) for 20 minutes at RT. Smears were air-dried and re-hydrated in 1xPBS for 5 minutes, rinsed briefly in distilled water, and incubated with pre-warmed (37°C) trypsin solution (0.1% in calcium chloride 0.1% pH 7.8) for 15 minutes in humidity chamber. Next, smears were washed in PBS for 5 minutes with gentle agitation, rinsed in distilled water and air dried. Smears were then incubated at 37°C with primary antibodies against hemoglobin (1:100, ab92492, Abcam) and human amylin (1:100, SC-377530, Santa Cruz Biotech) in humidity chamber for 30-40 minutes. They were next washed, air-dried and covered with anti-mouse IgG and anti-rabbit IgG secondary antibodies for 20-30 minutes at 37°C. After incubation smears were washed with PBS, rinsed in water, air dried and mounted in mounting media.

[00191] These sections of paraffin embedded pancreatic tissues were co-stained with Thioflavin S and an anti-amylin antibody.

[00192] Enzyme-linked immunosorbent assay (ELISA)

[00193] ELISA assays for human amylin (EZHA-52K, Millipore, MA; EIA-AMY-5, Raybiotech, GA), erythropoietin (EPO; 442807, Biolegend, CA), hypoxia-inducible transcription factor 1 α (HIF1- α ; MBS764727, MyBioSource, CA), hypoxia-inducible transcription factor 2 α (HIF2- α ; MBS2601406, MyBioSource, CA), arginase 1 (MBS289817, MyBioSource, CA), arginase 2 (MBS7216305, MyBioSource, CA), and von Hippel-Lindau (vHL; MBS288882, MyBioSource, CA) were performed according to the manufacturer's protocols.

[00194] Arginase activity assay

[00195] Arginase activity was measured in kidney homogenates using a colorimetric assay (MAK112, Sigma, MO). To eliminate urine from kidney samples, 300 μ L of kidney homogenate were filtered through a 10kD spin column (88513, ThermoFisher, MA) with centrifugation at 15000g for 60 min, 4°C. Collected remnant was used for experiments. Assay

was performed according to the manufacturer's protocol. Briefly, filtered kidney samples were incubated with reaction substrate mix for 2 hours at 37° C. The stop solution was applied, followed by incubation at 37° C for 35 min. Absorbance reading was taken at 430 nm. Arginase activity was calculated based on manufacturer's analysis instruction.

[00196] Immunoblot

[00197] Western blot analysis was performed on plasma, WBC lysates, and RBC lysates from humans using a primary antibody against amylin (1:2,000, T-4157, Bachem-Peninsula Laboratories, CA).

[00198] Immunohistochemistry

[00199] Immunohistochemical staining was performed on rat kidney slices using antibodies against amylin (1:200, SC-377530, Santa Cruz, TX), collagen IV (1:1000, ab6586, Abcam, MA), IBA-1 (1:200, 019-19741, Wako, VA), and EDI (1:100, MCA341GA, Biorad, PA). Biotinylated anti-mouse (1:400, BA2000, Vector lab, CA), AP conjugated anti-mouse and anti-rabbit IgG (1:50, A3562, A3687, Sigma, MO) were the secondary antibodies. The staining area for amylin was analyzed by ImageJ. The imaging area is 1280 x 1024 pixels; 1 pixel² is 0.053 μm^2 for 40X objective lens.

[00200] Statistical analysis

[00201] Statistical differences between groups were determined using student's t-test, one-way ANOVA or two-way ANOVA, as appropriate. Data are presented as mean $\pm$ standard error. Differences between groups were considered significant when $P < 0.05$. For human data, the homogeneity of variance was tested using Levene's test. Equal variance was assumed when $P > 0.05$ and normal t-test (parametric test) was applied. When ANOVA assumptions were violated, Mann-Whitney test (non-parametric test) was applied.

[00202] Generated Antibody Immunohistochemistry

[00203] Thin sections of paraffin-embedded tissues were incubated with purified amylin and A β primary antibodies. After washings, sections were incubated with HRP-conjugated anti-rabbit IgG secondary antibodies. The sections were then stained with AEC chromogen, mounted and imaged with Nikon light microscope. HIP rat pancreas was used as a positive control while AKO rat pancreas was used as negative control.

[00204] Generated Antibody Western Blot

[00205] For western blot, brain tissue was homogenized in homogenization buffer with protease and phosphatase inhibitor cocktail. Brain and Pancreas lysate were generated by centrifugation ($12,000 \times g$) of brain homogenate and collection of the supernatant fraction. After electrophoresis, blotting, and blocking, membranes were incubated with primary antibodies for amylin and Abeta. The specific staining of protein bands were verified by comparing the bands with commercially available antibody on same samples.

[00206] Generated Antibody ELISA

[00207] 96 well plates were coated with Amylin or A β or samples in bicarbonate buffer (pH 9.6) overnight at 4°C. After three washes with PBST (Tween 0.05%) plates were blocked with 300 μ l of blocking solution followed by three more washes. Then plates were incubated with 100 μ l of detection antibody (A β or Amylin) for 1 hour at room temperature. Again plates were washed three times followed by incubation with 100 μ l of secondary antibody. After washing three times with PBST plates were incubated with TMB substrate. After getting signal reaction was stopped by 50 μ l of stop solution and read at 450 nm. The signal in HIP rat pancreas which was used as a positive control while AKO rat pancreas as negative control.

[00208] RESULTS

[00209] Example 1: Humans with type-2 diabetes have amylin deposition in RBCs.

[00210] The relationship between HbA1c level (the common marker of hyperglycemia) and amylin concentration in RBC lysates from patients with type-2 diabetes or diseases that are commonly associated with insulin resistance was assessed, including heart failure, cancer and stroke. Western blot analysis of matched plasma, RBC lysate and white blood cell (WBC) lysate from a human with type-2 diabetes (the positive control for amylin dyshomeostasis) detected both monomeric amylin (Fig. 18 A) and amylin-positive higher molecular weight bands (Fig. 24). RBC lysates from individuals with a primary diagnosis of type-2 diabetes (T2D) (without heart failure, cancer or stroke) had higher amylin concentration than those from healthy individuals (H) and patients with type-1 diabetes (T1D group; the negative control for amylin) (Fig. 18B). Patients with a primary diagnosis of heart failure (HF), cancer (C), or stroke (S) also had elevated RBC amylin levels independent of type-2 diabetes as a secondary diagnosis (Fig. 18B). Table 3 describes the average amount of amylin (ng/g) in each patient population erythrocytes as depicted in FIG. 18B. Lighter colored symbols in the HF and C groups indicate heart failure or cancer without type-2

diabetes. RBC amylin and HbA1c levels were highly variable in all groups (Fig. 18C-I), except in patients with HF and type-2 diabetes in whom higher HbA1c levels correlated with RBC amylin accumulation. There were non-significant inverse correlations between HbA1c and RBC amylin levels in the HF without diabetes (Fig. 18F), cancer with diabetes (Fig. 18G) and stroke (Fig. 18I) groups.

	Healthy	T2D	HF-T2D	HF	C-T2D	C	S	T1D
Avg Amylin (ng/g)	1.20035	1.987502	2.751755	2.100945	2.457987	1.761388	2.800678	1.36667

Table 3: Average amount of amylin (ng/g) in each patient population

[00211] These results suggest that type-2 diabetes and diseases associated with insulin resistance such as heart failure, cancer and stroke promote amylin accumulation in RBCs in humans.

[00212] Example 2: Amylin deposition in RBCs results from hypersecretion of amyloid-forming human amylin.

[00213] The HIP rat is a unique animal model for late-life onset type-2 diabetes as it is characterized by pancreatic expression of the human (amyloid-forming) variant of amylin, whereas other rodent models for type-2 diabetes express only the native, non-amyloid forming amylin. As in humans, the development of type-2 diabetes in HIP rats is associated with pancreatic amyloid (Fig. 25). the amylin content in RBCs, WBCs, and plasma from diabetic HIP rats and non-diabetic WT littermates (Fig. 19A) was compared. Amylin was concentrated in RBCs and the *difference* in amylin levels between WT and diabetic HIP rats was greater in RBCs than in plasma or WBCs (Fig. 19A).

[00214] Analysis of freshly packed RBCs by flow cytometry (Fig. 19B) and amylin ELISA (Fig. 19C) revealed a correlation between RBC amylin levels and different levels of non-fasted blood glucose (normal, ≤ 11 mM, 6-8 months old; prediabetic, 11-14 mM on two consecutive measurements separated by >3 days, 10-12 months old; and diabetic, >14 mM on two consecutive measurements, separated by >3 days; 14-16 months old).

[00215] Confocal microscopy analysis of RBCs that were double-stained for amylin and hemoglobin showed amylin deposition on RBCs from HIP rats (Fig. 19D). Amylin appeared to co-localize with glycophorin A (Fig. 19E), a membrane protein that is specific to RBCs, suggesting amylin accumulation on the RBC membrane. The analysis of RBCs co-stained for amylin and glycophorin A using super-resolution imaging (STORM) showed the presence of amylin within the RBC membrane with some dense patches on the outer part of

the cell membrane (Fig. 19F). Electron microscopic examination of immunogold-labeled thin sections of epoxy resin-embedded RBCs indicated the presence of amylin within the cell membrane (FIG. 26; arrowhead pointing to amylin deposits).

[00216] The results demonstrate that hypersecretion of the amyloid-forming human variant of amylin leads to amylin deposition within circulating RBCs.

[00217] Example 3: Amylin-coated RBCs have lower deformability and functional (non-glycated) hemoglobin.

[00218] Next, pathophysiological characteristics of RBCs from diabetic HIP rats and RBCs from diabetic rats without amylin dyshomeostasis (UCD rats) and non-diabetic WT rats, as they express only the non-amyloid forming rat amylin was investigated. Compared to RBCs from WT rats, RBCs from HIP rats contained less functional (non-glycated) hemoglobin (FIG. 20A), whereas there was no difference between UCD and WT rats. RBCs of HIP, UCD and WT rats (~ 12 months old rats) had similar oxygen dissociation curves (FIG. 20B) indicating that the affinity of hemoglobin for oxygen, the release of bound oxygen and the partial oxygen pressure for maintaining oxygen saturation are not significantly affected by amylin dyshomeostasis or hyperglycemia.

[00219] In flow cytometry experiments, the distribution of the forward scattering (FSC) signals by RBCs from WT rats (FIG. 20C; lowest peak) was bimodal, which reflects the biconcave disk shape of normal RBCs. In the case of amylin-coated RBCs from diabetic HIP rats, the FSC distribution was monomodal (FIG. 20C; highest peak), indicating changes in RBC morphology towards a more spherical shape. The Pearson coefficient of dissymmetry (PCD), which indicates the departure from sphericity, suggested distinct morphological changes in RBCs from HIP rats compared to WT and UCD rats (FIG. 20C, middle peak). Incubation of RBCs from WT rats with aggregated human amylin for 4 hours (as described in previous studies) replicated the morphological change observed in HIP rat RBCs (FIG. 27). Altered shape of RBCs from HIP and UCD rats did not affect the response to an osmotic resistance test, as the propensity for hemolysis in hypo-osmotic solutions was comparable for RBCs from all three rat groups (FIG. 20D).

[00220] These results show that amylin deposition in RBCs is associated with lower hemoglobin concentrations and reduced RBC deformability, independently of effects of chronic glucose levels. At an early stage of diabetes, the cumulative effects of amylin

dyshomeostasis and hyperglycemia (as in HIP rats) or hyperglycemia alone (as in UCD rats) do not appear to induce significant changes in hemolysis and oxygen dissociation.

[00221] Example 4: The microvasculature is disrupted in kidneys of rats with amylin dyshomeostasis.

[00222] Next the structural integrity of the capillary network and the stability of the capillary beds in kidneys from age- and blood glucose-matched HIP and UCD rats was investigated. immunohistochemistry with antibodies against amylin (brown, dark stain) and collagen IV (Col IV; green, light stain) were used, a component of the basement membrane, to anatomically localize amylin deposition with respect to the vasculature. In HIP rat kidneys, there were patches of amylin deposits in arterioles, interstitial tissue between the tubules (FIG. 20E) and glomeruli (FIG. 20F). Vascular amylin deposition correlated with accumulation of macrophages, as indicated by co-staining for amylin and the ionized calcium-binding adapter molecule 1 (IBA1), a marker of macrophage activation (FIG. 20G). There were macrophages in areas of amylin deposition, which may indicate a potential role for these cells in the clearance of vascular amylin deposition. Staining with ED1 (FIG. 20H), an antibody against the cluster of differentiation (CD) 68 protein (that is highly expressed by circulating macrophages), supports the increased activity of macrophages in areas of vascular amylin deposition. In contrast, vascular amylin deposits and macrophage infiltration were not found in kidneys from diabetic UCD rats.

[00223] These data suggest that amylin dyshomeostasis injures the capillaries, which may be associated with systemic inflammatory responses leading to macrophage infiltration that may exacerbate ischemic vascular injury in HIP rats.

[00224] Example 5: Amylin dyshomeostasis activates renal hypoxia signaling pathways.

[00225] The kidney is a critical component of a regulatory feedback loop that controls the hematocrit via EPO production. Both HIP and UCD rats had renal dysfunction, as indicated by polyuria and albuminuria (FIG. 28A and 28Bb). Creatinine clearance was elevated in diabetic HIP rats compared with WT littermates and diabetic UCD rats (FIG. 28C). The plasma level of EPO, the hormone that signals an increased demand for RBCs to the bone marrow, was higher in diabetic HIP rats than in WT littermates (FIG. 21A) and tended to be higher in age-matched diabetic UCD rats vs. WT rats ($P = 0.27$). The reticulocyte count was also higher in HIP than in WT rats (FIG. 21B). Despite elevated

plasma EPO levels, the average hematocrit was not different in HIP rats compared with WT littermates, but was lower in HIP compared to UCD rats (FIG. 21C). The spleen, a major blood reservoir, had lower weight in diabetic HIP rats compared to age-matched WT rats (FIG. 29). Compared with diabetic UCD rats and healthy WT rats, diabetic HIP rats had elevated levels of HIF-1 α and HIF-2 α in whole kidney tissue homogenates (FIG. 21D and 21E). Consistent with elevated HIF-2 α , HIP rat kidneys also had upregulated arginase-1 and 2 proteins (FIG. 21F and 21G) and greater arginase activity (FIG. 21H). Increased stabilization of HIF α units in HIP rat kidney tissue correlated with a trend towards downregulation of the von Hippel-Lindau (vHL) tumor suppressor protein (FIG. 30) suggesting impaired degradation of HIF α units.

[00226] These results suggest that activation of hypoxia signaling in kidneys and downstream upregulation of EPO are associated with pathologic erythropoiesis and amylin deposition in RBCs.

[00227] Example 6: Blocking of adhesion proteins in endothelium reverses amylin dyshomeostasis and HIF activation.

[00228] The adhesion of RBCs to cultured vascular endothelial cells (ECs) under flow condition tended to be greater for HIP rat RBCs than RBCs from UCD rats ($P = 0.17$) and from WT rats ($P = 0.17$) (FIG. 22A). Greater adhesion of RBCs from HIP rats to ECs was found in experiments without flow condition (FIG. 22B) in an adhesion test in which RBCs from WT rats that were incubated with 50 μ M synthetic human amylin for 4 hours is shown in FIG. 22C. Analysis of kidney capillary lysates by Western blot (A) and ELISA (B) showed a trend towards upregulation of the expression of vascular cell adhesion molecule 1 (VCAM-1) in HIP rats (FIG. 31). Taken together, these results suggest that the cell membrane adhesion proteins may play a role in amylin accumulation at the RBC-capillary interface. To test this hypothesis, EETs were used, which are primarily expressed by vascular ECs and RBCs and are known to downregulate the expression of VCAM-1 in endothelium. *Ex vivo* incubation with ($\pm$)14(15)-EET reduced the adhesion of HIP rat RBCs to cultured endothelial cells (FIG. 22D). Upregulation of EETs by treatment with an inhibitor of soluble epoxide hydrolase, the enzyme that degrades endogenous EETs, was associated with lower amylin deposition in renal microvasculature (the HIP-T group; FIG. 22E). The treatment lowered renal accumulation of HIF-2 α (FIG. 22F) and HIF-1 α (FIG. 22G) and had variable effects on arginase expression and arginase activation (FIG. 32) in HIP rat kidneys.

[00229] Taken together, these results indicate that 1) reduced capillary RBC flux owing to amylin deposition on RBCs and the capillary wall likely contributes to tissue hypoxia in HIP rats and 2) EETs reduce this effect by downregulation of adhesion proteins in the vascular endothelium.

[00230] Example 7: Transfused amylin-coated RBCs upregulate EPO through HIF-2 α activation in kidneys.

[00231] RBCs act as both oxygen carriers and mediators of oxygen sensing and signaling pathways within ECs. To determine whether amylin deposition on RBCs activates hypoxia signaling pathways in tissues, amylin-coated RBCs from HIP rats were administered to WT rats. Rats were given 300 μ l freshly packed RBCs/day for seven days. WT rats given similar volumes of RBCs from diabetic UCD rats were used as positive controls for the possible effects of hyperglycemia on RBC function, whereas WT rats receiving RBCs from WT rats served as negative controls. Plasma levels of EPO were elevated in WT rats receiving amylin-coated RBCs from HIP rats compared to WT rats that received either RBCs from diabetic UCD rats or WT rats (FIG. 23A). Kidneys of WT rats receiving HIP rat RBCs had increased stabilization of HIF-1 α (FIG. 23B) but no difference in HIF-2 α levels (FIG. 23C), compared with rats in the two control groups.

[00232] To examine further a possible ‘amylin stress’ on oxygen-sensing pathways, aggregated human amylin (hA) (0.08 μ g/g body weight) was intravenously administered to diabetic UCD rats, daily, for one week. Intravenous infusion of aggregated human amylin in UCD rats led to amylin deposition on RBCs (FIG. 23D). This acute ‘amylin stress’ provoked an increase in plasma EPO level (FIG. 23E; the ‘after hA inj’ rat group) and no difference in renal HIF-2 α and HIF-1 α levels in whole kidney tissue homogenate (FIG. 23F). Arginase 1 protein levels were elevated in kidney tissue homogenates from amylin-infused UCD rats, whereas there was no significant change of arginase 2 protein expression (FIG. 23G).

[00233] These data indicate elevated EPO and accumulation of HIFs in kidneys as a direct response to circulating amylin-coated RBCs (FIG. 23H).

[00234] Example 8: ELISA showed high titers in collected bleeds

[00235] Direct ELISA was done to check the titer between the 1st (prebleed), 2nd, 3rd, 4th and final bleed which were collected from the different time point from the rabbits. First the comparison between the 1st (Prebleed) and 2nd bleed for the titer 1:100, 1:1000 and 1:10000 (Fig. 33 A) was shown. First bleed (Prebleed) showed O.D equals to blank suggest

none of any antibody present in the rabbit before immunization. The comparison between the 2nd and 3rd bleed for the titer 1:10000, 1:100000 and 1:1000000 was determined. Bleed 3rd was found to be higher titer as compared to bleed 2nd (Fig. 33 B). Similarly comparison between the 3rd and 4th bleed for the titer 1:10000, 1:100000 and 1:1000000. Bleed 4th was found to be higher titer as compared to bleed 3rd (Fig. 33 C). Finally, the comparison for final bleed for the titer 1:10000, 1:100000 and 1:1000000 were determined. Final titer for Amylin N-terminal, C-terminal and A β N-terminal, C-terminal antibody was obtained at 1:100000 (Fig. 33 D).

[00236] Example 9: Direct ELISA showed purified antibodies are specific for their antigen

[00237] (Fig. 34 A) Showed the direct ELISA for Amylin in pancreas homogenate of HIP rat (overexpressing human amylin in pancreas) and AKO rat (Amylin knockout rat) with Amylin N-terminal and C-terminal antibody. HIP served as a positive control and AKO served as a negative control. Both antibodies detect higher signal in HIP as compared to AKO. (Fig. 34B) Showed the direct ELISA for A β in brain homogenate of AD rat (overexpressing human A β in brain) and Wild type (WT) rat with A β N-terminal and C-terminal antibody. AD served as a positive control. Both antibodies detect higher signal in AD as compared to WT. Fig. 34 C shows a diagram of how one embodiment of the presently described ELISA functions.

[00238] Example 10: Custom ELISA with antibodies showed similar results with commercially available ELISA

[00239] The Direct ELISA was performed in Plasma of HIP mouse (overexpressing Human IAPP), WT mouse and AKO mouse (Amylin is knockout). Higher signals in HIP mouse Plasma as compared with WT and AKO mouse Plasma (Fig. 35, left panel). At the same time with the same set of samples, the sandwich ELISA was performed with commercially available kit (Fig 35, right panel). Similar results were found with the Direct ELISA as was found with commercially available ELISA Kit that confirm the present antibody is specific and sensitive for ELISA.

[00240] Example 11: Immunohistochemistry showed antibodies are specific for their proteins

[00241] Immunohistochemistry was performed using Amylin N-terminal, C-terminal and A β N-terminal antibody in their positive and negative tissue samples sections. Both N

terminal and C-terminal antibodies against amylin showed positive signal in HIP pancreas but not in AKO pancreas (arrows) (Fig. 36A). Similarly antibody against A β N-terminal showed positive signal in AD brain sections but not in negative control (WT rat heart) (Fig. 36B).

[00242] Example 12: A β N-terminal and C-terminal antibodies specifically detect the A β peptides in western blot

[00243] A β N-terminal and A β C-terminal antibodies detect the Anaspec A β synthetic peptide in western blot at below 10 KD (Figs. 37A & B).

[00244] Example 13 Immunogens and method used to create amylin and A β antibodies.

[00245] FIGs. 38A-B shows the design and creation of antibodies against amylin and amyloid β peptide. FIG. 38 A. The peptides used to generate antibodies against amylin and A β . The peptides are also sknown as the following sequence numbers: Amylin peptide (SEQ ID NO: 2); Amylin C (SEQ ID NO: 3); Amylin N (SEQ ID NO: 4); Amyloid β peptide (SEQ ID NO: 5); A β N (SEQ ID NO: 6); and A β C (SEQ ID NO: 7). FIG. 38 B. shows injection and bleed collection schedule.

[00246] Example 14: Keyhole limpet hemocyanin1 (Fragment) KLH sequence used to generate polyclonal antibodies of the invention herein is listed below and referred to as SEQ ID NO: 1.

[00247] LSVRLIVVLALANAENLVRKSVEHLTQEETLDLQAALRELQMDSSSI
 GFQKIAAAHGAPASCVHKDTSIACCIHGMPTFPWHRAYVVHMERALQTKRRTSGL
 PYWDWTEPITQLPSLAADPVYIDSQGGKAHTNYWYRGNIDFLDKKTNRAVDDRLFE
 KVKPGQHTHLMESVLDALQDEFCKFEIQFELAHNAIHLYLVGGKHDYSMANLEYTA
 YDPIFFLHHSNVDRIFAIWQRLQELRNKDPKAMDCAQELLHQKMEPFSWEDNDIPLT
 NEHSTPADLFDYCELHYDYDTLNLNGMTPEELKTYLDERSSRARAFASFRLKGFSGS
 ANVFVYVCIPDDNDRNDDHCEKAGDFFVLGGPSEMKWQFYRPYLFDLSDTVHKMG
 MKLDGHYTVKAELFSVNGTALPDDLLPHPVVVHHPEKGFTDPPVKHHQSANLLVR
 KNINDLTREEVLNLREAFHKFQEDRSVDGYQATAEYHGLPARCPRPDAKDRYACCV
 HGMPIFPWHRLFVTQVEDALVGRGATIGIPYWDWTEPMTHIPGLAGNKTYVDSHG
 ASHTNPFHSSVIAFEENAPHTKRQIDQRLFKPATFGHHTDLFNQILYAFEQEDYCDFE
 VQFEITHNTIHAWTGGSEHFSMSSLHYTAFDPLFYFHHSNVDRWLAVWQALQMRR
 HKPYRAHCAISLEHMHLPFAFSSPLNNNEKTHANAMPNKIYDYENVLHYTYEDLT

FGGISLENIEKMIHENQQEDRIYAGFLLAGIRTSANVDIFIKTTSVQHKAGTFAVLGG
SKEMKWGFDRVFKFDITHVLKDLDLTADGDFEVTVDITEVDGTKLASSLIPHASVIR
EHARVKFDKVPRSLIRKNVDRLSPEEMNELRKALALLKEDKSAGGFQQLGAFHGE
PKWCPSPEASKKFACCVHGMSVFPWHRLTLVQSENALRRHGYDGALPYWDWTSP
LNHLPELADHEKYVDPEDGVEKHNPWFDGHIDTVDKTTTRSVQNKLFEPFGHYT
SIAKQVLLALEQDNFCDFEIQYEIAHNYIHALVGGAQPYGMASLRYTAFDPLFYLLHH
SNTDRIWAIWQALQKYRGKPYNVANCAVTSMREPLQPFGLSANINTDHVTKEHSVP
FNVFDYKTNFNIEYDTLEFNGLSISQLNKKLEAIKSQDRFFAGFLLSGFKKSSLVKFN
ICTDSSNCHPAGEFYLLGDENEMPWAYDRVFKYDITEKLHDLKLHAEDHFYIDYEVF
DLKPASLGKDLFKQPSVIHEPRIGHHEGEVYQAEVTSANRIRKNIENLSLGELESLRA
AFLEIENDGTYESIAKFHGSPGLCQLNGNPISCCVHGMPTFPWHRLYVVVVENALL
KKGSSVAVPYWDWTKRIEHLPHLISDATYYNSRQHHYETNPFHHGKITHENEITTRD
PKDSLFSDFYFYEQVLYALEQDNFCDFEIQLEILHNALHSLGKGKYSMSNLDYAA
FDPVFFLHHATTDRIWAIWQDLQRFRKRPYREANCAIQLMHTPLQPFDKSDNNDEAT
KTHATPHDGFYQNSFGYAYDNLELNHYSIPQLDHMLQERKRHDRVFAFLLHNIG
TSADGHVFCVCLPTGEHTKDCSHEAGMFSILGGQTEMSFVFDRLYKLDITKALKKNG
VHLQGDFFDLEIEITAVNGSHLDSHVIHSPTILFEAGTDSAHTDDGHTEPVMIRKDITQL
DKRQQLSLVKALESMKADHSSDGFQAIASFHALPPLCPSPAASKRFACCVHGMATFP
QWHRLYTVQFQDSLRLKHGAVVGLPYWDWTLPRSELPELLTVSTIHDPETGRDIPNPF
IGSKIEFEGENVHTKRDINRDLFQGSTKTHHNWFIEQALLALEQTNYCDFEVQFEIM
HNGVHTWVGGEKPYGIGHLHYASYDPLFYIHHSQTDRIWAIWQSLQRFRGLSGSEA
NCAVNLMKTPLKPFSGAPYNLNDHTHDFSKPEDTFDYQKFGYIYDTLEFAGWSIRG
IDHIVRNRQEHSRVFAFLLLEGFGTSATVDFQVCRTAGDCEDAGYFTVLGGGEKEMP
WAFDRLYKYDITETLDKMNLRHDEIFQIEVTITSYDGTVLDSGLIPTPSIYDPAHHDIS
SHHLSLNKVRHDLSTLSERDIGSLKYALSSLQADTSADGFAAIASFHGLPAKCNDSH
NNEVACCIHGMPTFPWHRLYTLQFEQALRRHGSSVAVPYWDWTKPIHNIPHLFTD
KEYYDVWRNKVMPNPFARGYVPSHDTYTVRDVQEGFLHSTSTGEHSALLNQALLA
LEQHDYCDFAVQFEVMHNTIHYLVGGPQVYSSLHYASYDPIFFIHSFVDKVWA
VWQALQEKRLPSDRADCAVSLMTQNMRFHYEINHNQFTKKHAVPNDVFKYELL
GYRYDNLEIGGMNLHEIEKEIKDKQHHRVFAFLLHGIRTSADVQFQICKTSEDCH
HGGQIFVLGGTKEMAWAYNRLFKYDITHALHDAHITPEDVFHPSEPFFIKVSVTAVN
GTVLPASILHAPTIIYEPGLDHHEDHHSSSMAGHGVRKEINTLTAEVDNLKDAMRA
VMADHGPNGYQAIAAFHGNPPMCPMPDGKNYSCCTHGMATFPWHRLYTKQMED
ALTAHGARVGLPYWDGTTAFTALPTFVTDEEDNPFHHGHIDYLGVDTTTRSPRDKLF

NDPERGSESFFYRQVLLALEQTDFCQFEVQFEITHNAIHSWTGGLTPYGMSTLEYTTY
 DPLFWLHHANTDRIWAIWQALQEYRGLPYDHANCEIQAMKRPLRPFSDPINHNAFT
 HSNAPKPTDVFEYSRNFQYDNLRFHGMTIKKLEHELEKQKEEDRTFAAFLHGIKKS
 ADVSFDVCNHDGECHFAGTFAILGGEHEMPWSFDRLFRYDITQVLKQMHLEYDSDF
 TFHMRIDTSGKQLPSDLIKMPTVEHSPGGKHHEKHHEDHHEDILVRKNIHSLSHHEA
 EELRDALYKLQNDESHGGYEHIAGFHGYPNLCPEKGDEKYPCCVHGMSIFPHWHRL
 HTIQFERALKKHGSHLGIPYWDWTQTISSLPTFFADSGNNNPFFKYHIRSINQDTV RD
 VNEAIFQQTKFGEFSSIFYLALQALEEDNYCDFEVQYEILHNEVHALIGGAEKYSMST
 LEYSAFDPYFMIHHASLD

[00248] DISCUSSION

[00249] Hypersecretion of human amylin is associated with amylin deposition in the microvasculature and RBCs leading to impaired RBC-capillary interaction and activation of hypoxia signaling pathways. Impairment of tissue oxygen-sensing found in diabetic HIP rats was mirrored in control rats that were given amylin-coated RBCs intravenously and in diabetic UCD rats that were given human amylin intravenously. These results indicate that amylin-coated RBCs are a trigger of hypoxia signaling pathways. These deleterious effects result in part from a reduced flux of amylin-coated RBCs through the capillaries that may involve adhesion proteins. Blocking the expression of adhesion proteins in the vascular endothelium by upregulation of EETs reduces amylin dyshomeostasis and HIF activation.

[00250] All publications, patents, and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication, patent, or patent application was specifically and individually indicated to be incorporated by reference, including the references set forth in the following list:

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153. Peake M, Whiting M. Measurement of serum creatinine- current status and future goals. *Clin Biochem Rev.* 2006;27:173-184.
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[00251] It will be understood that various details of the presently disclosed subject matter can be changed without departing from the scope of the subject matter disclosed herein. Furthermore, the foregoing description is for the purpose of illustration only, and not for the purpose of limitation.

CLAIMS

1. A method of detecting amylin in a patient, said method comprising:
 - a. obtaining a blood sample from a human patient;
 - b. detecting whether amylin is present in the erythrocytes by conducting an amylin ELISA; and
 - c. using the amylin ELISA to determine the amount of amylin in the patient's erythrocytes.
2. The method of claim 1, and further comprising:
treating the patient by increasing circulating Epoxyeicosatrienoic acids (EETs) if the amount of amylin in the patient's erythrocytes is higher than the amount of amylin in healthy control erythrocytes.
3. The method of claim 1, and further comprising:
diagnosing the patient with pre-diabetes if the amount of amylin in the patient's erythrocytes is higher than the amount of amylin in healthy control erythrocytes and lower than the amount of amylin in diabetic control erythrocytes; and
treating the patient with lifestyle changes, biological, genetic, or pharmacological intervention suitable for the treatment of prediabetes.
4. The method of claim 3, wherein the patient is diagnosed with pre-diabetes when the amount of amylin in the erythrocytes is between about 1 and 2 ng/g total protein.
5. The method of claim 1, and further comprising:
diagnosing the patient with type-2 diabetes, if the amount of amylin in the patient's erythrocytes is higher than the amount of amylin in healthy control erythrocytes and pre-diabetic control erythrocytes; and
treating the patient with therapeutically effective amount of an anti-diabetic therapeutic.
6. The method of claim 5, wherein the patient is diagnosed with type-2 diabetes when the amount of amylin in the erythrocytes is greater than or equal to about 2 ng/g total protein.

7. The method of claim 1, and further comprising:
diagnosing the patient as at risk for developing a comorbidity of type-2 diabetes, if the amount of amylin in the patient's erythrocytes is higher than the amount of amylin in healthy control erythrocytes; and
treating the patient by increasing circulating Epoxyeicosatrienoic acids (EETs).
8. The method of claim 7, wherein circulating EETs are increased by administering an effective amount of an inhibitor of Soluble epoxide hydrolase.
9. The method of claim 7, wherein the patient is diagnosed with a comorbidity of type-2 diabetes when the amount of amylin in the erythrocytes is about 1.7 ng/g total protein or greater.
10. The method of claim 9, wherein the comorbidity is cancer.
11. The method of claim 10, and further comprising:
treating the patient with a therapeutically effective amount of an anti-cancer therapeutic.
12. The method of claim 9, wherein the comorbidity is heart failure.
13. The method of claim 12, and further comprising:
treating the patient with therapeutically effective amount of a heart failure therapeutic.
14. The method of claim 9, wherein the comorbidity is a stroke.
15. The method of claim 14, and further comprising:
treating the patient with therapeutically effective amount of a therapeutic for the treatment of stroke.
16. The method of claim 1, and further comprising:

diagnosing the patient with amylin related hypoxia if the amount of aggregated amylin in the patient's erythrocytes is higher than the amount of aggregated amylin in healthy control erythrocytes; and

treating the patient by increasing circulating Epoxyeicosatrienoic acids (EETs).

17. The method of claim 16, wherein the amylin related hypoxia is renal, cardiac, or brain hypoxia.

18. The method of claim 16, wherein circulating EETs are increased by administering an effective amount of an inhibitor of Soluble epoxide hydrolase.

19. A method to determine the effectiveness of biologic, lifestyle changes, or pharmaceutical intervention in preventing complications in a patient with type-2 diabetes or co-morbidity thereof, or amylin related renal hypoxia, said method comprising:

- a. obtaining a first blood sample from the patient diagnosed with type-2 diabetes or co-morbidity thereof, or amylin related renal hypoxia;
- b. using an aggregated amylin ELISA to determine the amount of aggregated amylin in the patient's erythrocytes;
- c. administering an effective amount of biologic, lifestyle change, or pharmacologic intervention;
- d. obtaining a subsequent blood sample from the patient;
- e. using the aggregated amylin ELISA to determine the amount of aggregated amylin in the patient's erythrocytes; and
- f. determining the intervention is effective if the amount of aggregated amylin in the erythrocytes in the subsequent sample is less than the amount of aggregated amylin in the erythrocytes in the first sample.

20. The method of any of claims 3, 5, 11, 13, or 15 wherein the treatment increases circulating Epoxyeicosatrienoic acids (EETs).

FIGURES

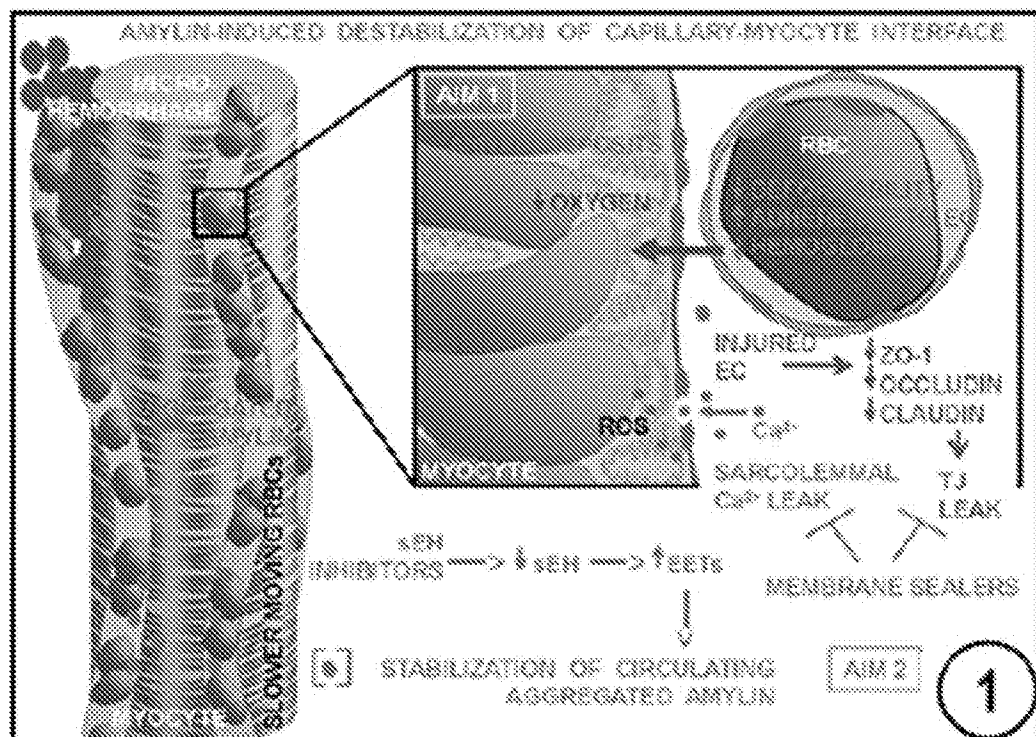


FIG. 1

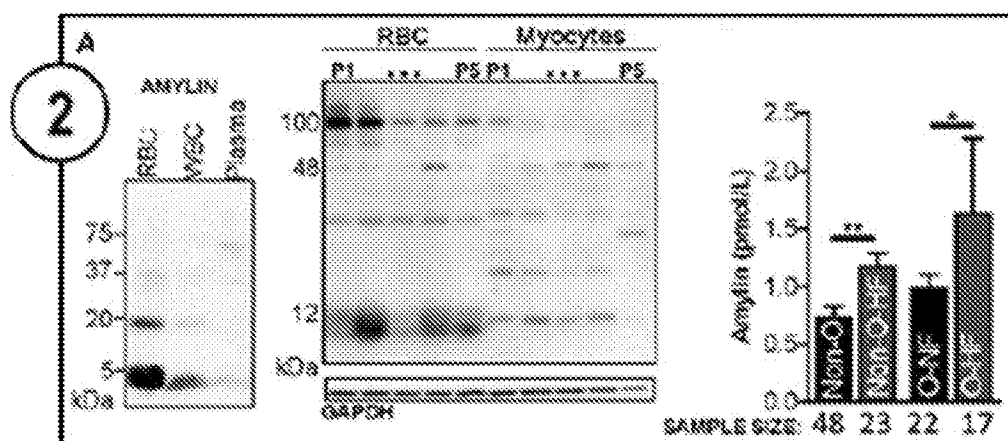


FIG. 2A

FIG. 2B

FIG. 2C

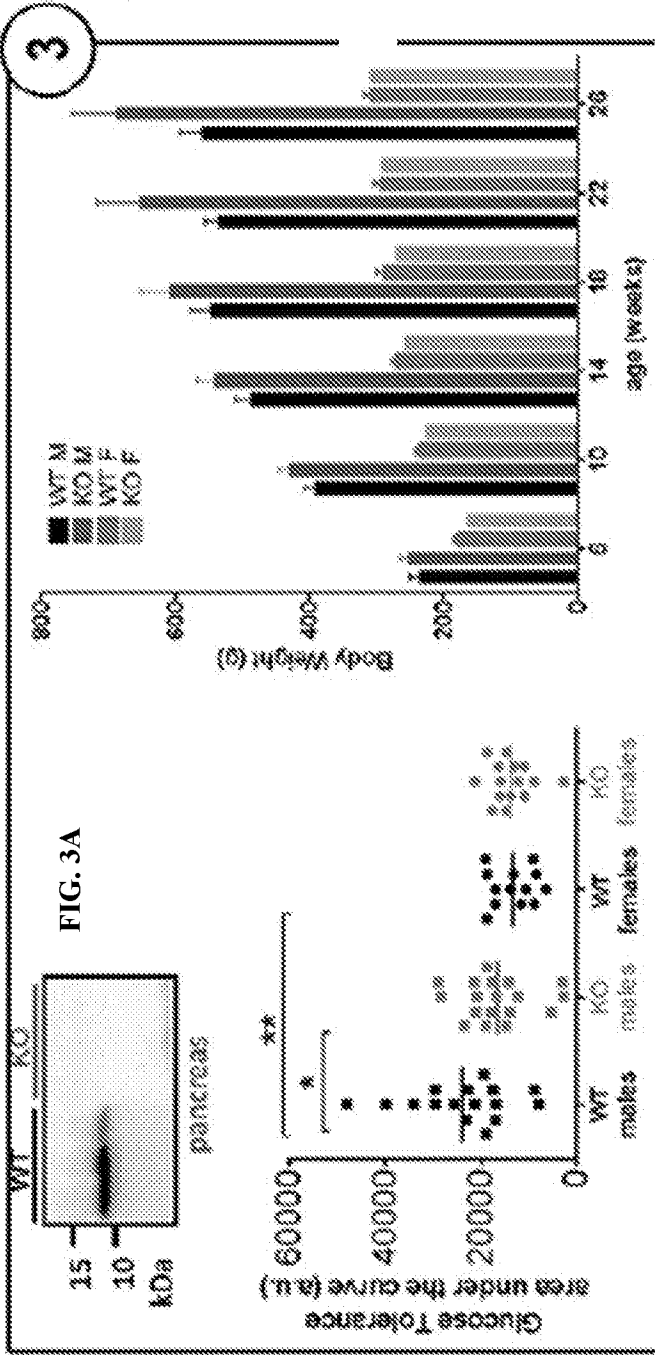
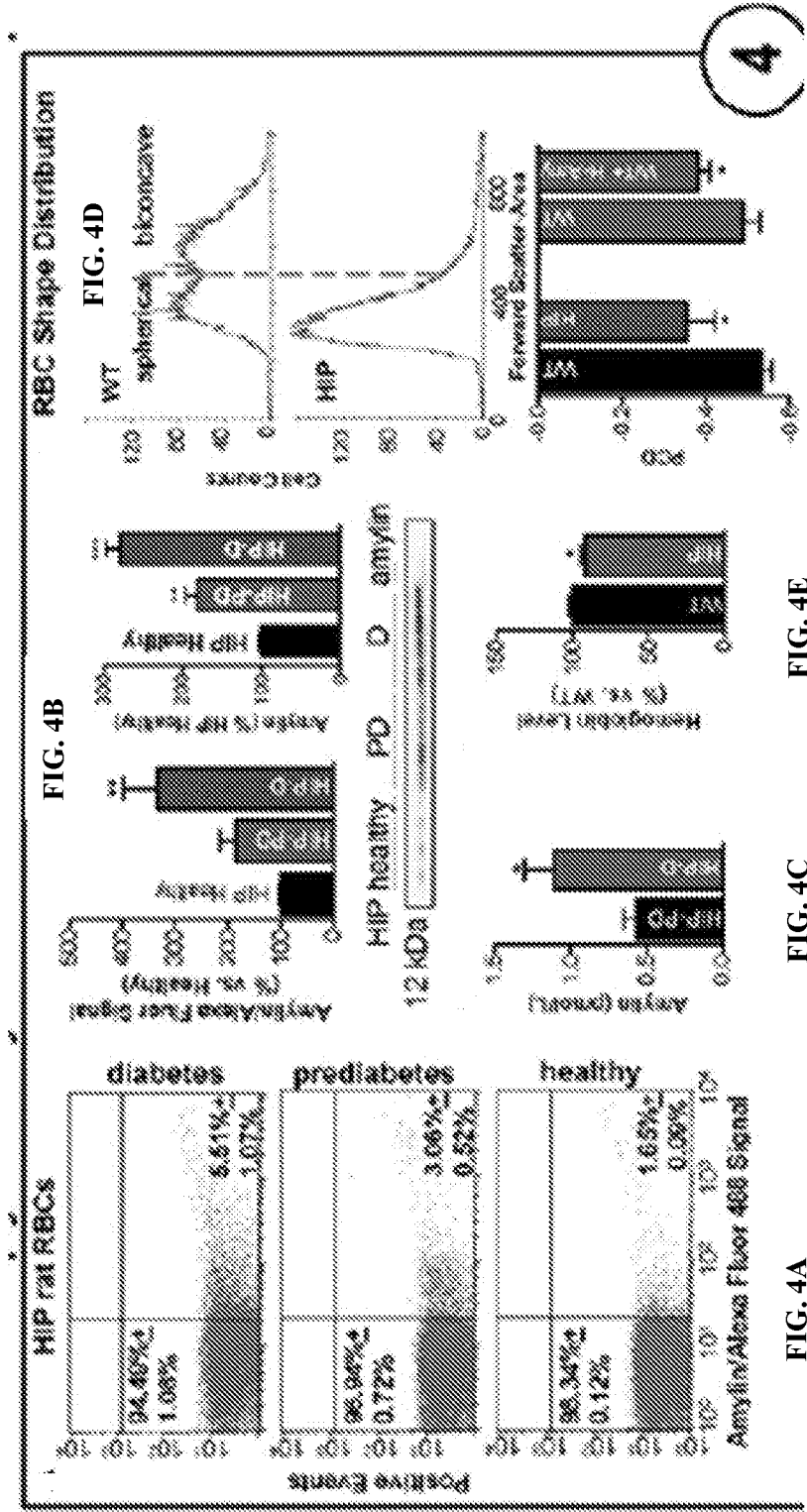
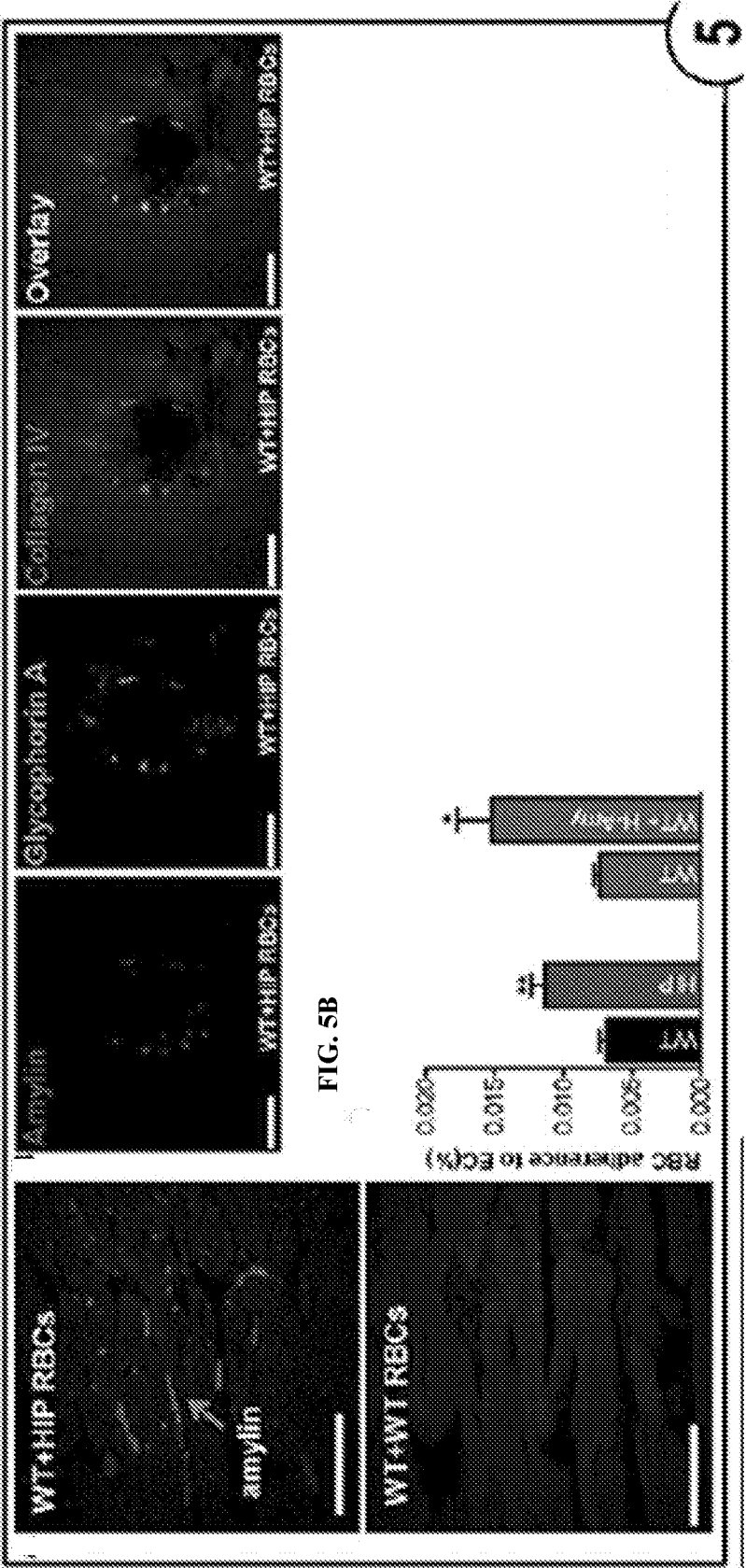


FIG. 3C

FIG. 3B





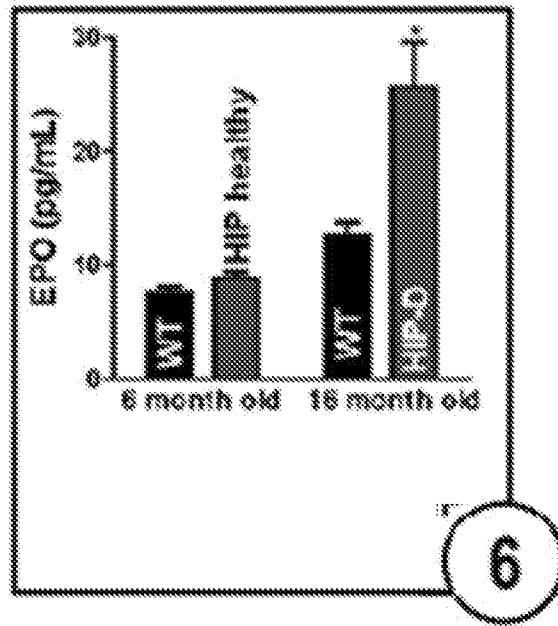


FIG. 6

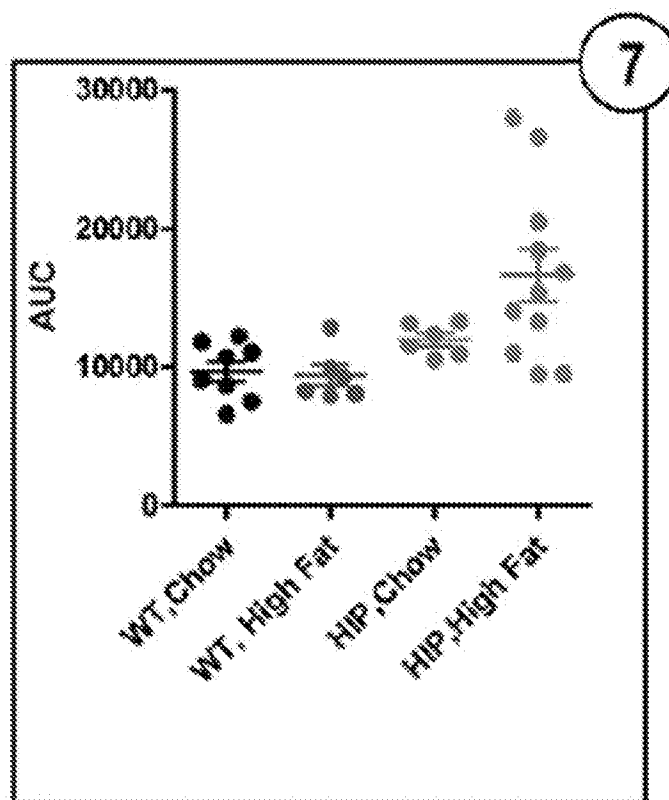


FIG. 7

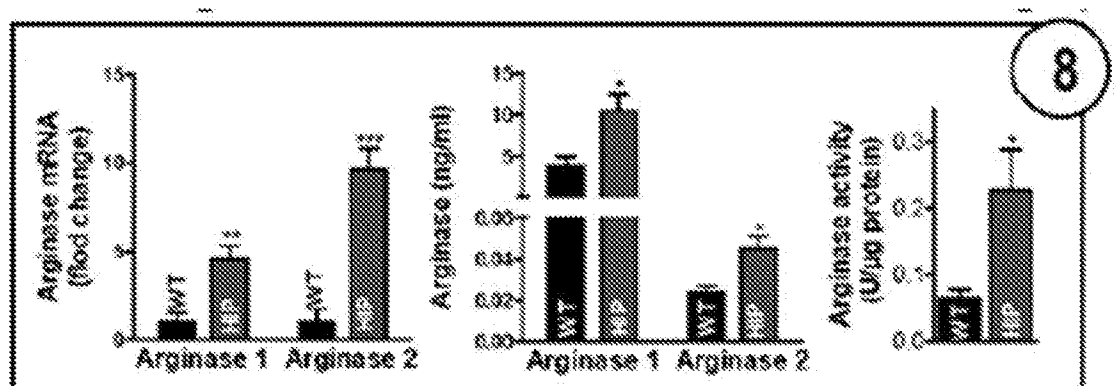
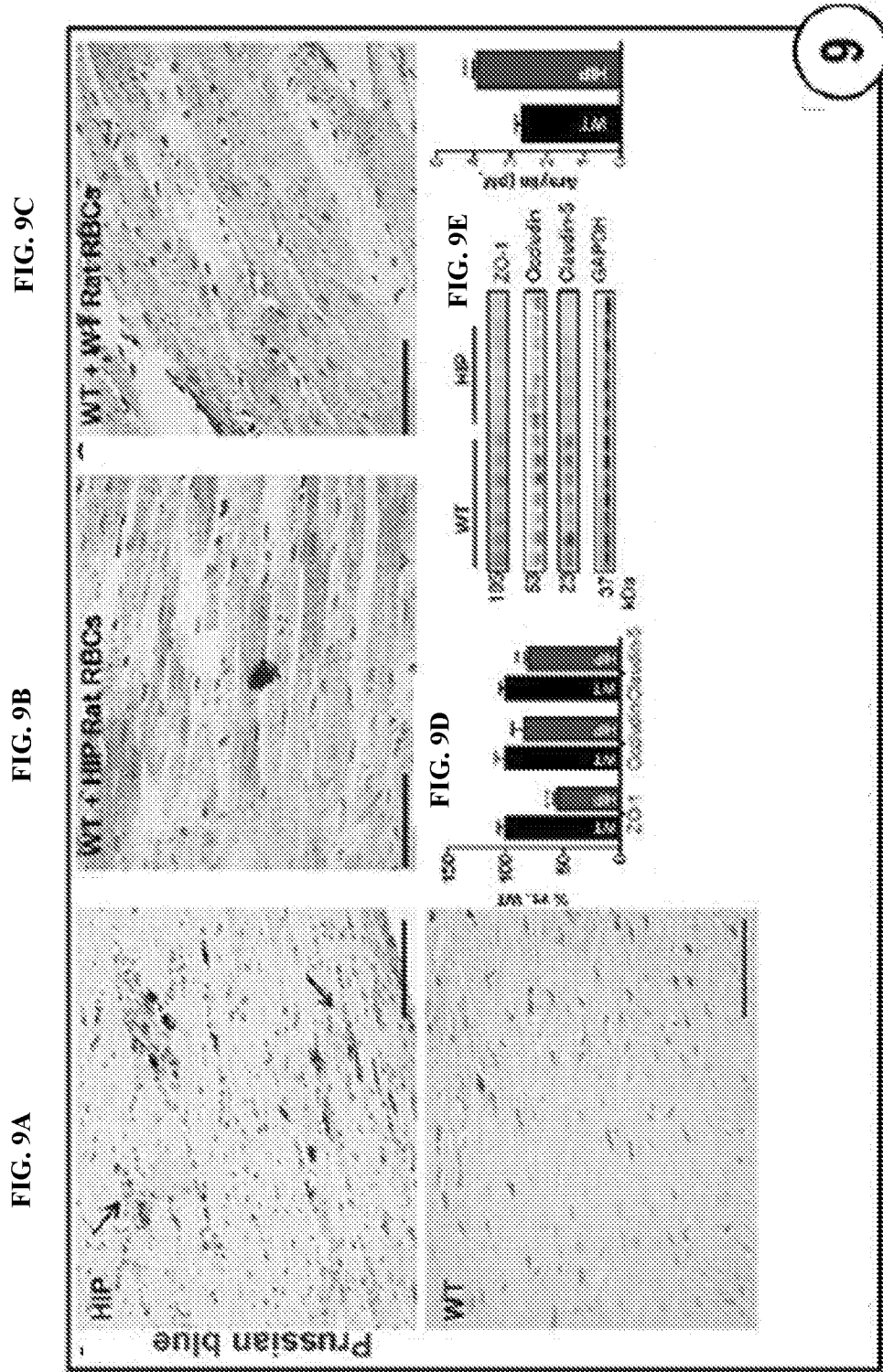


FIG. 8A

FIG. 8B

FIG. 8C



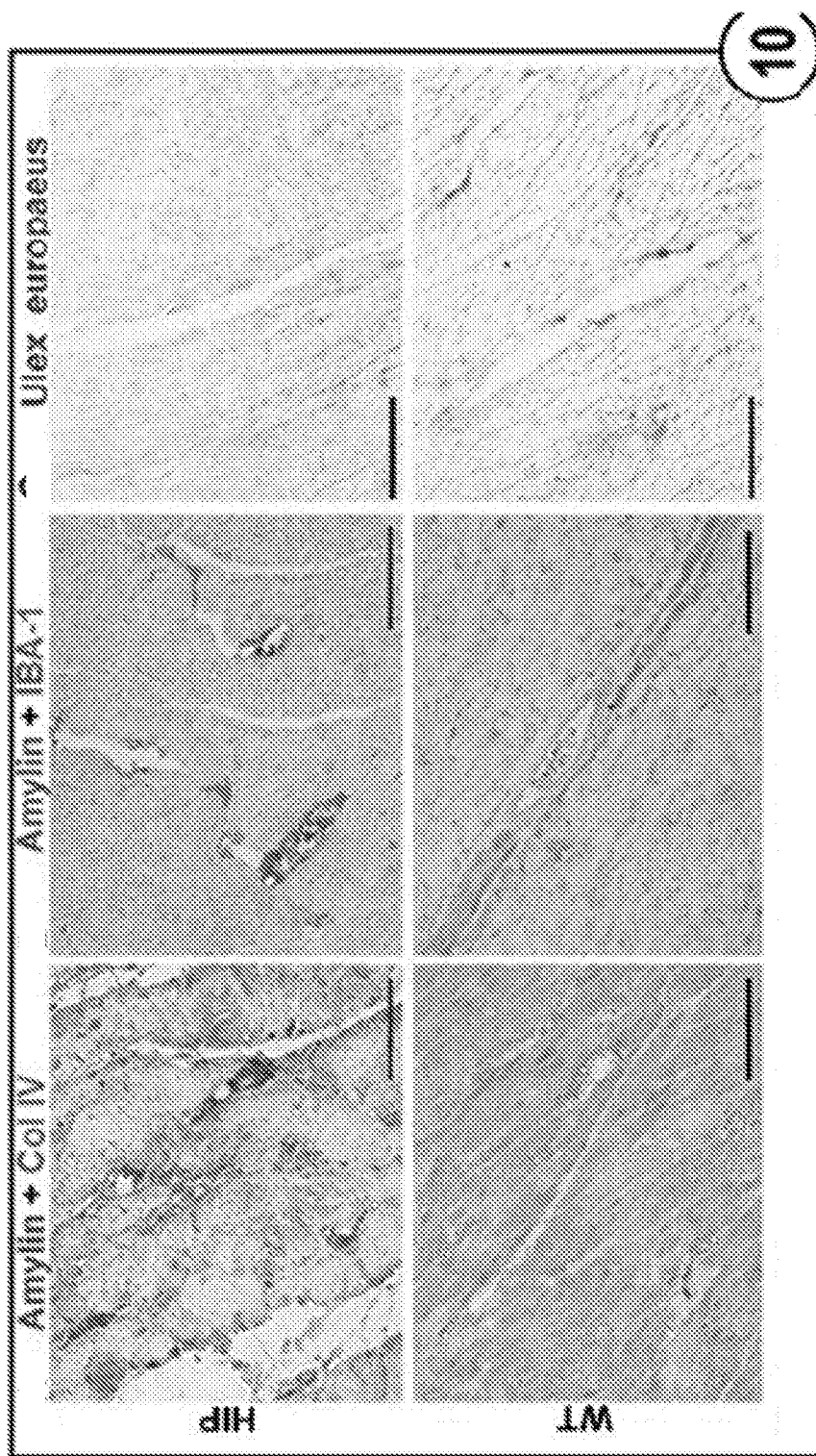
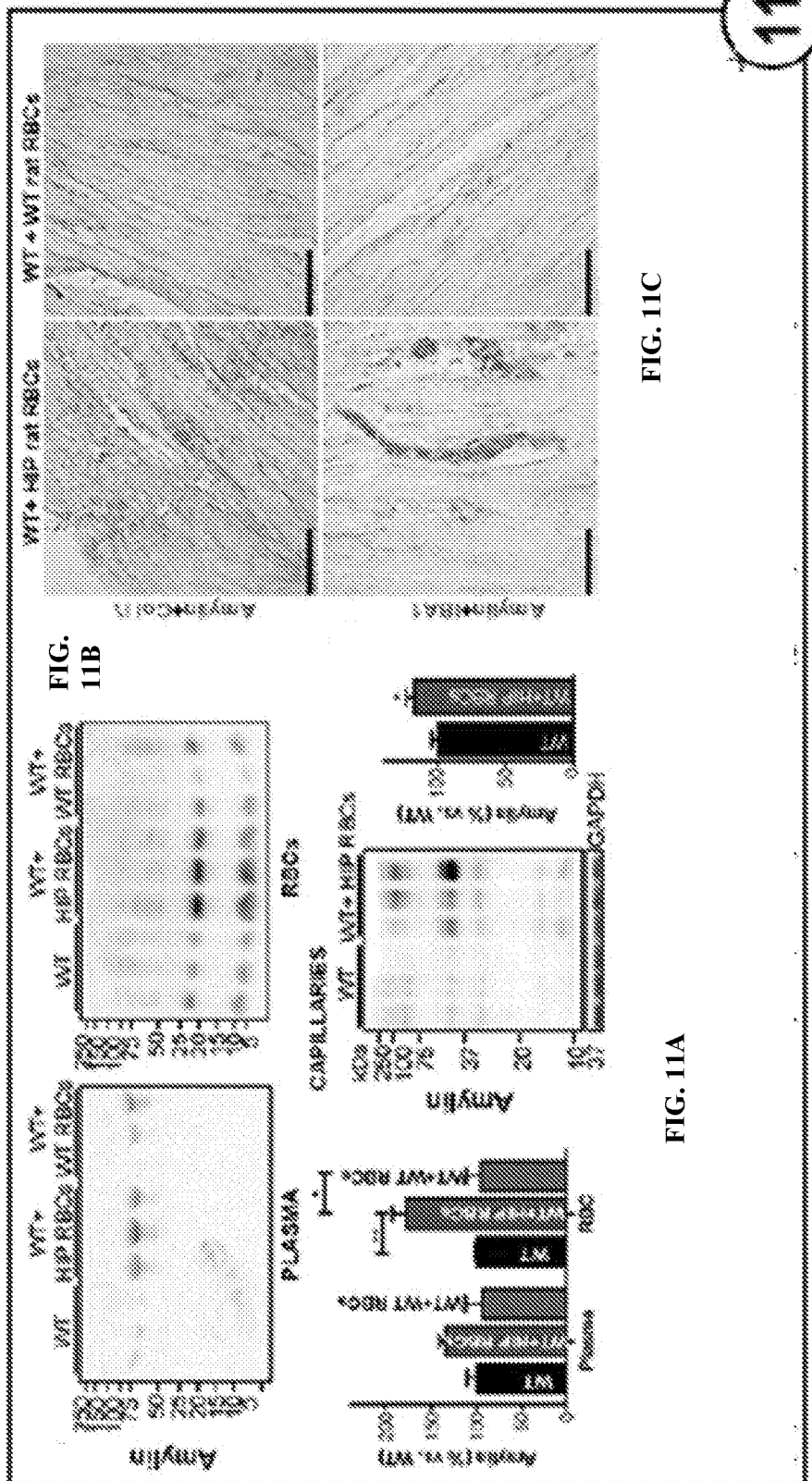


FIG. 10C

FIG. 10B

FIG. 10A



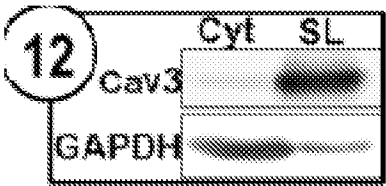


FIG. 12

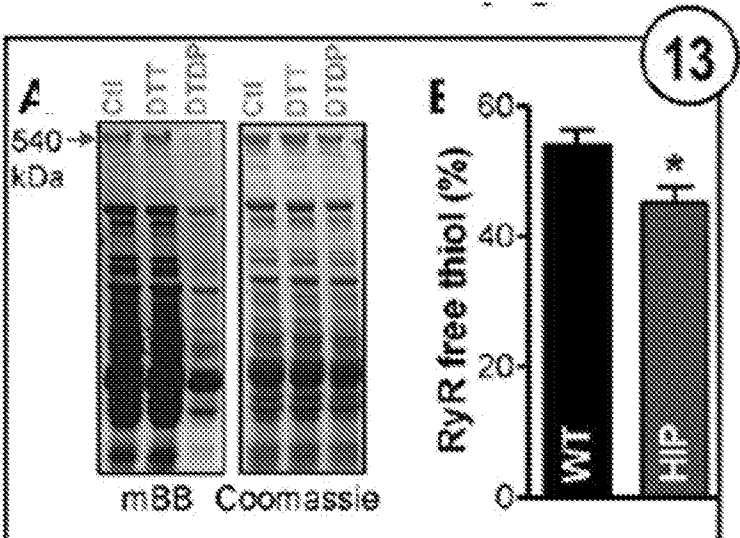


FIG. 13A

FIG. 13B

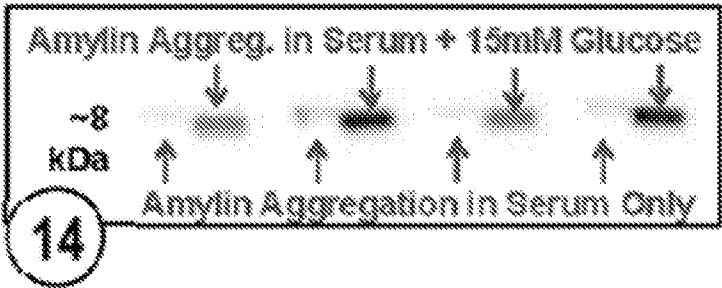
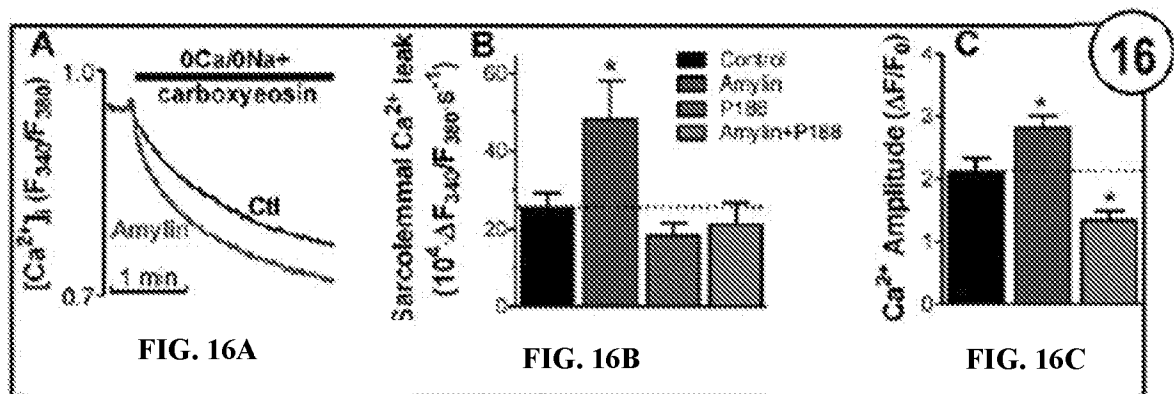
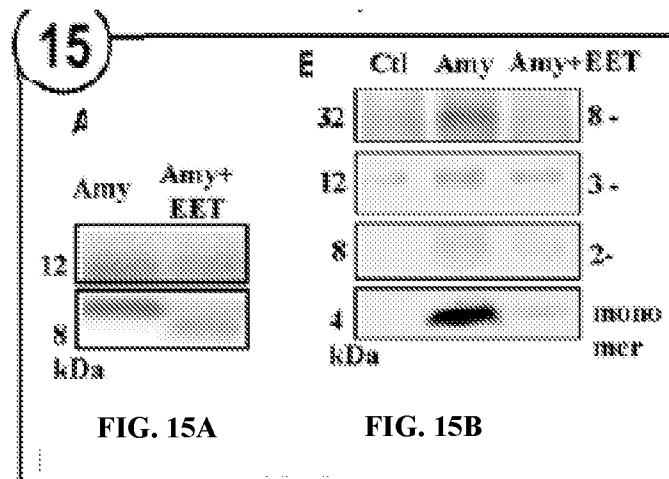


FIG. 14



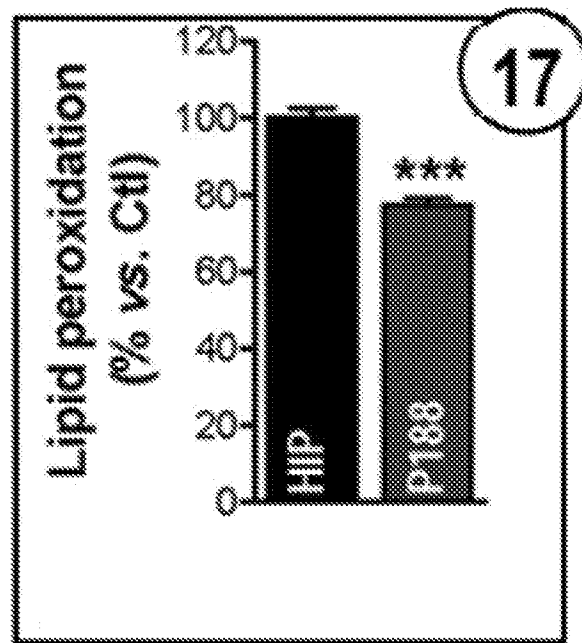


FIG. 17

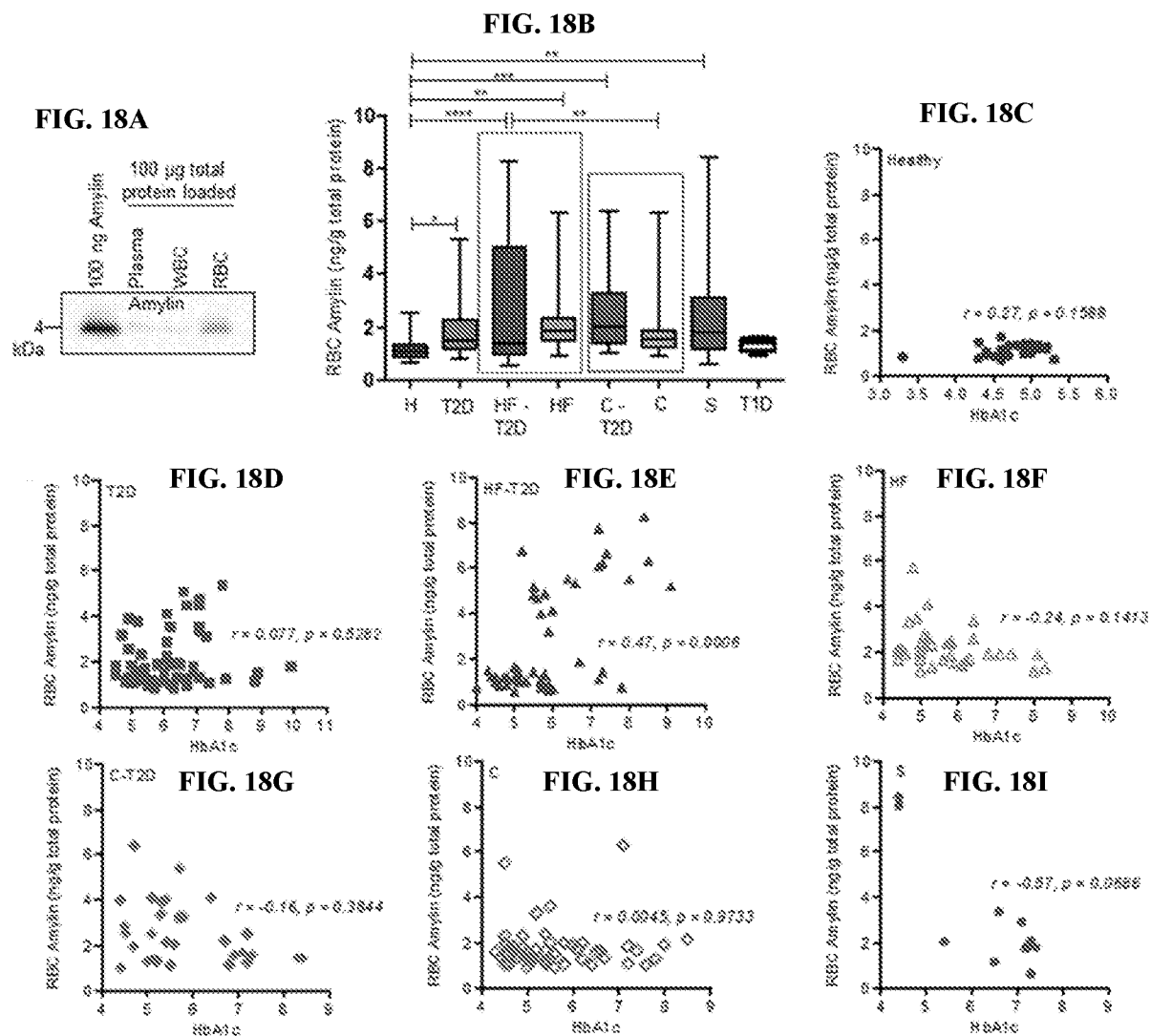


FIG. 19A

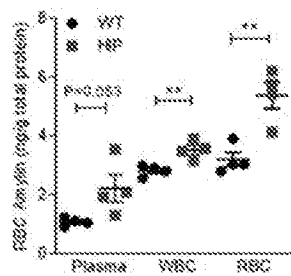


FIG. 19D

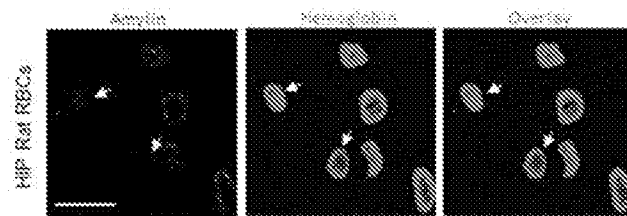


FIG. 19E

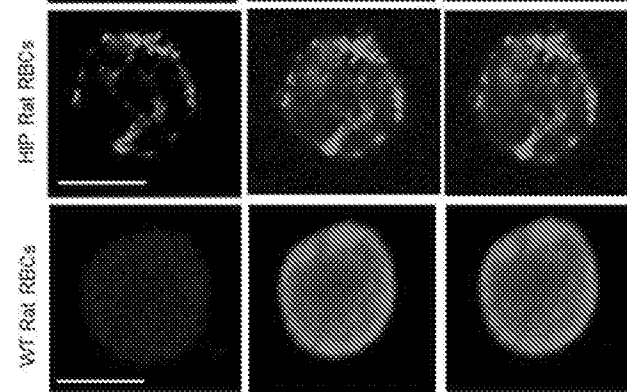


FIG. 19F

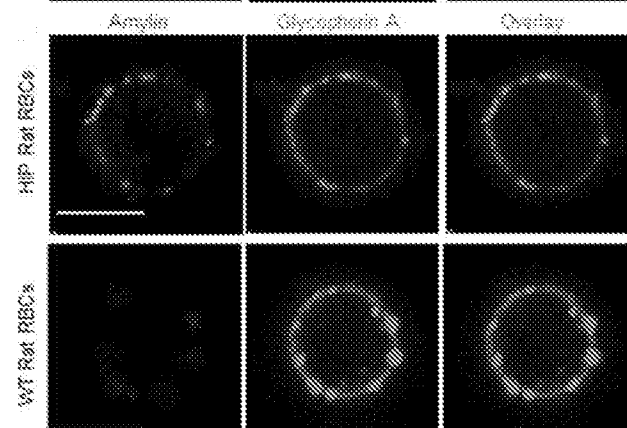


FIG. 19B

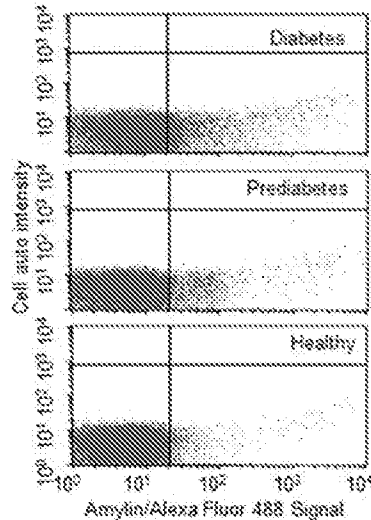


FIG. 19C

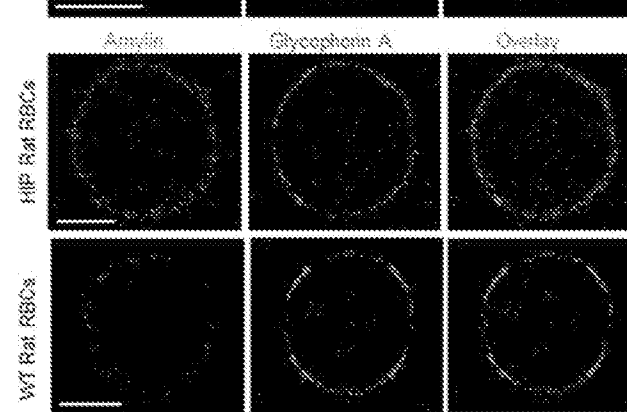
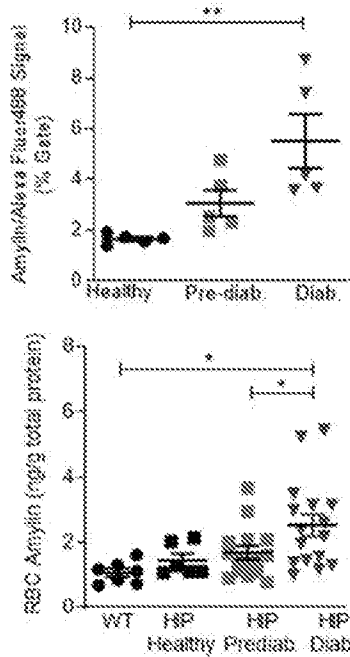


FIG. 20A

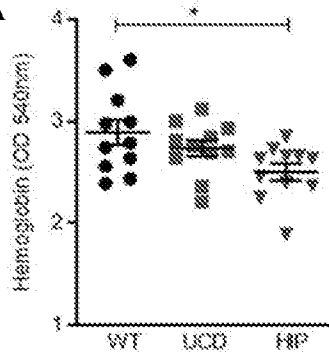


FIG. 20B

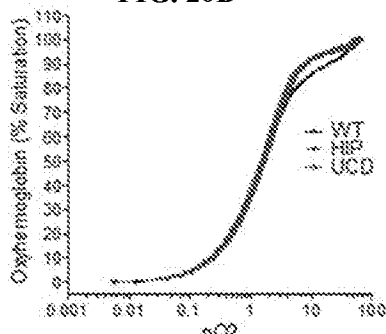


FIG. 20C

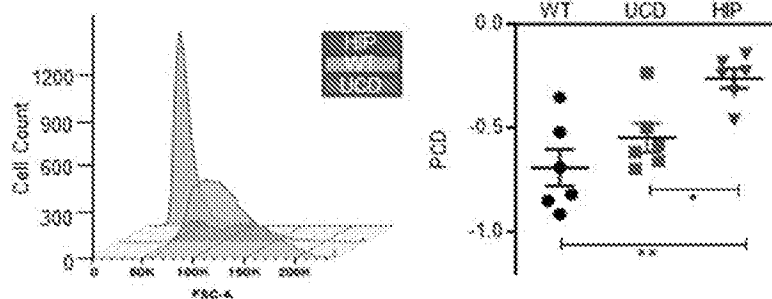


FIG. 20D

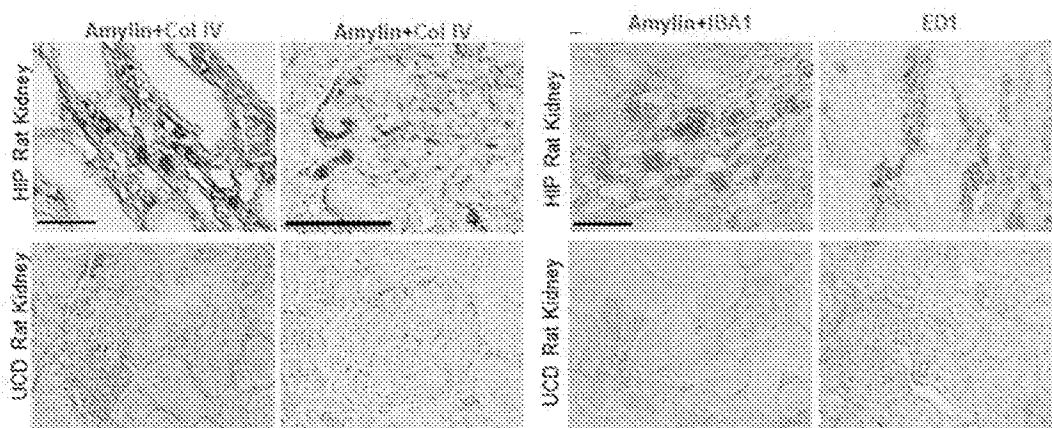
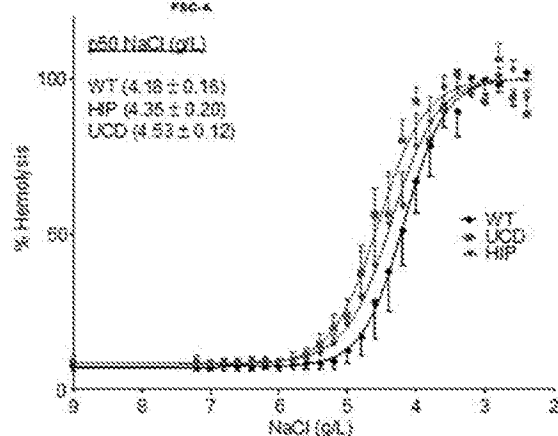


FIG. 20E

FIG. 20F

FIG. 20G

FIG. 20H

FIG. 21A

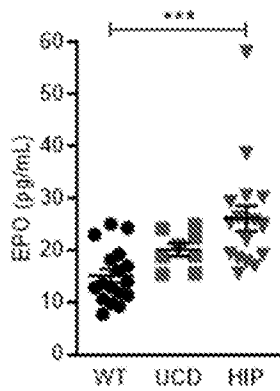


FIG. 21B

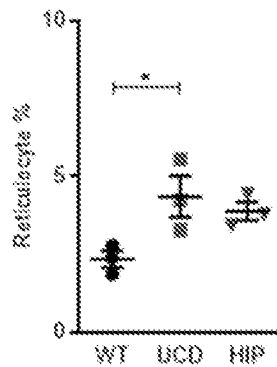


FIG. 21C

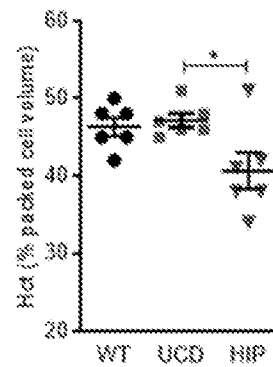


FIG. 21D

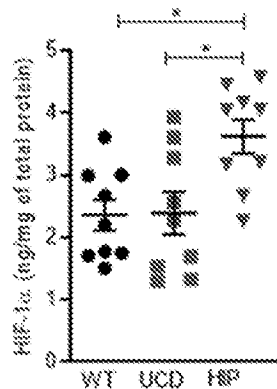


FIG. 21E

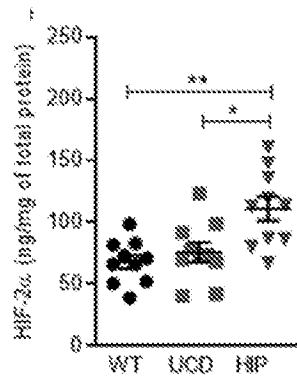


FIG. 21F

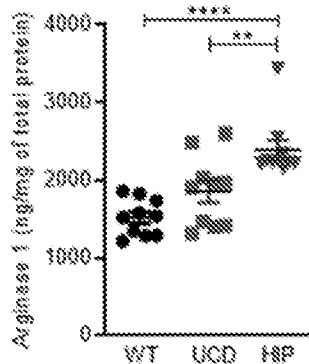


FIG. 21G

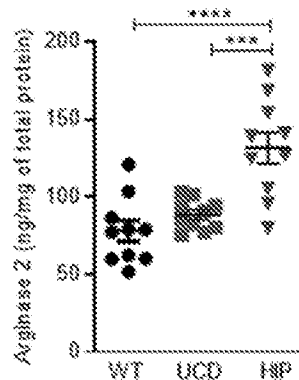
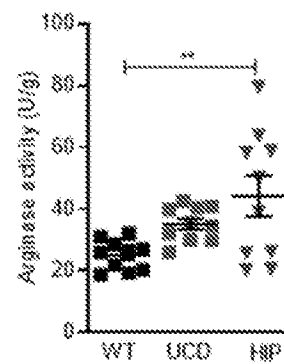
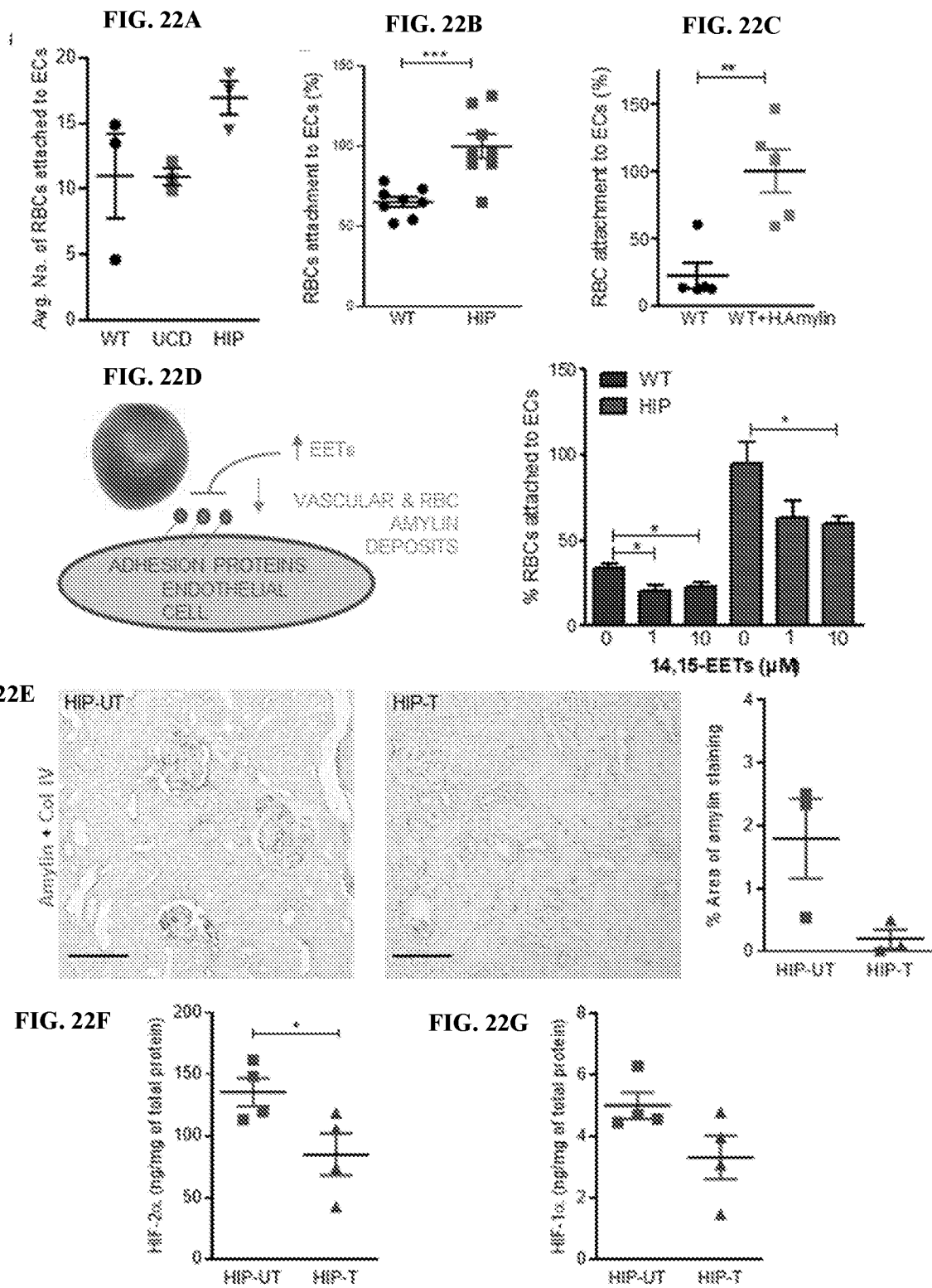


FIG. 21H





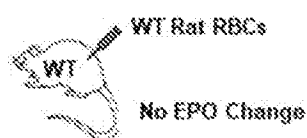


FIG. 23A

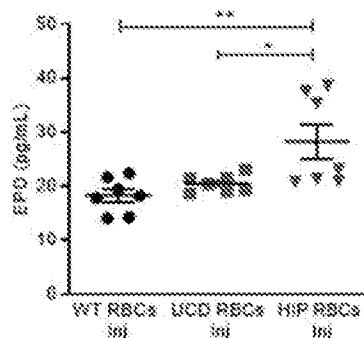


FIG. 23B

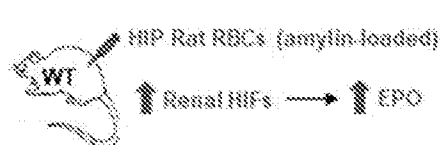
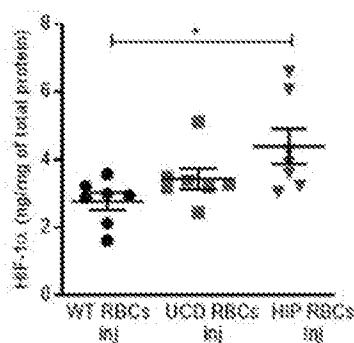


FIG. 23C

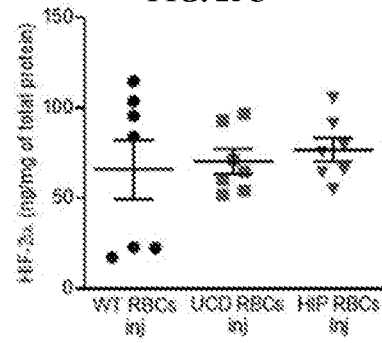


FIG. 23D

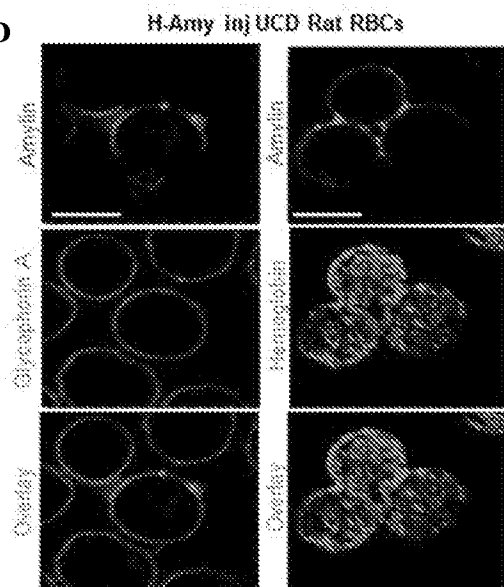


FIG. 23F

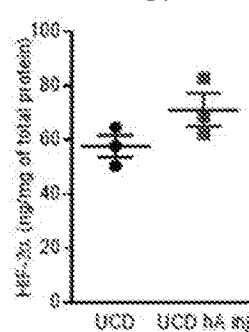


FIG. 23G

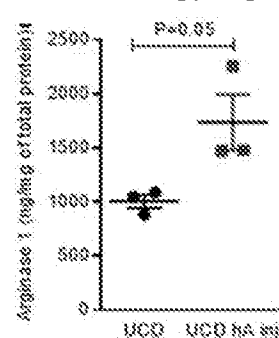


FIG. 23E

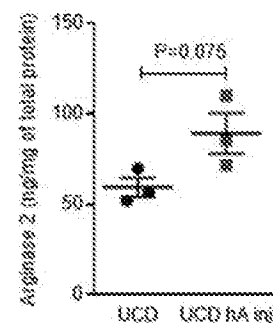
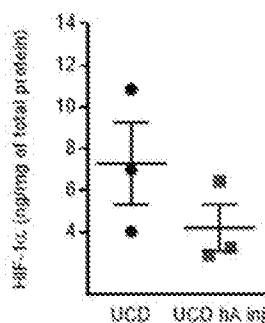
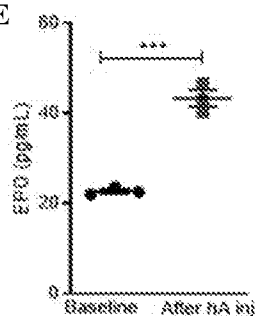
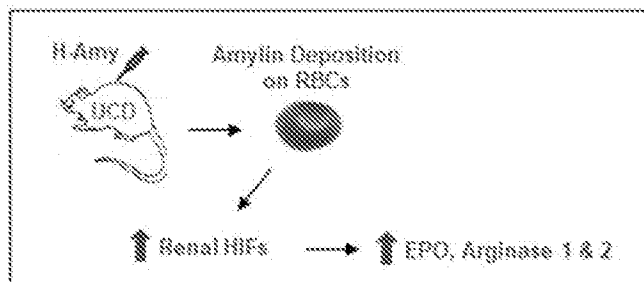


FIG. 23H



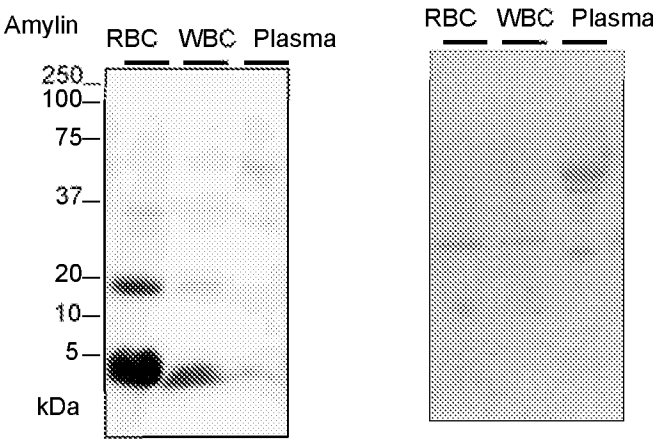


FIG. 24

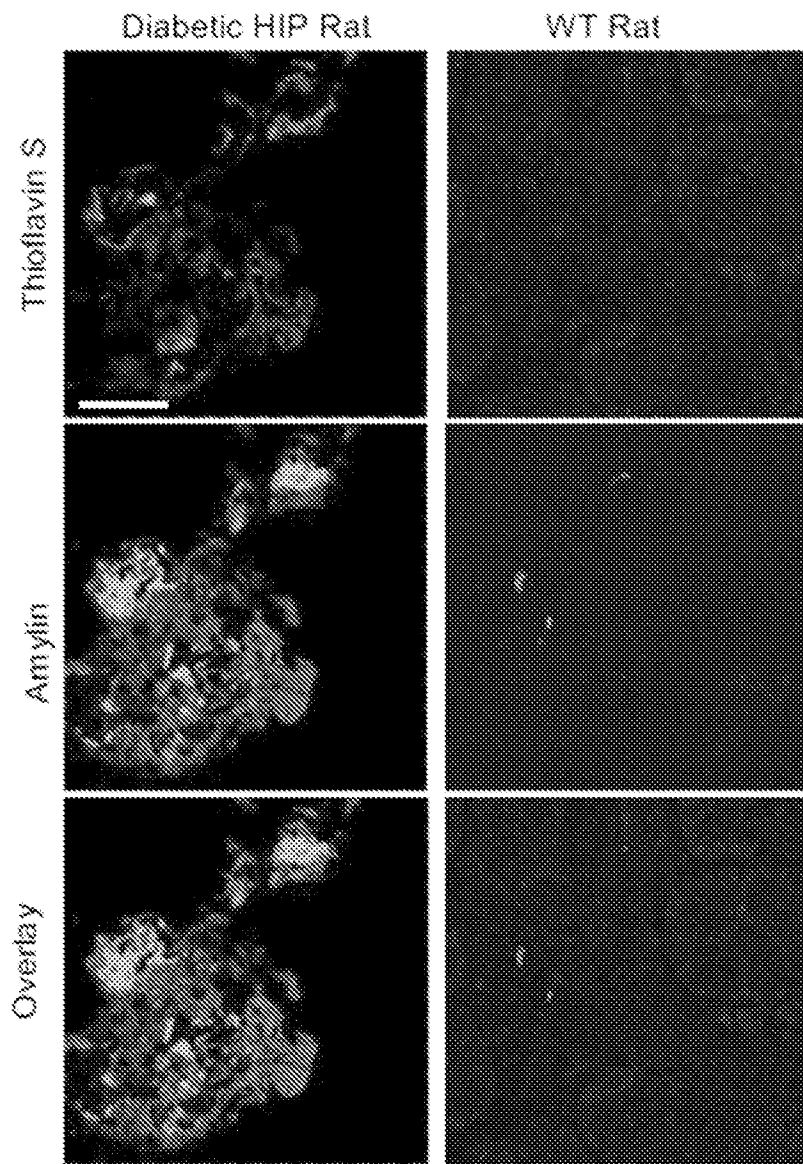


FIG. 25

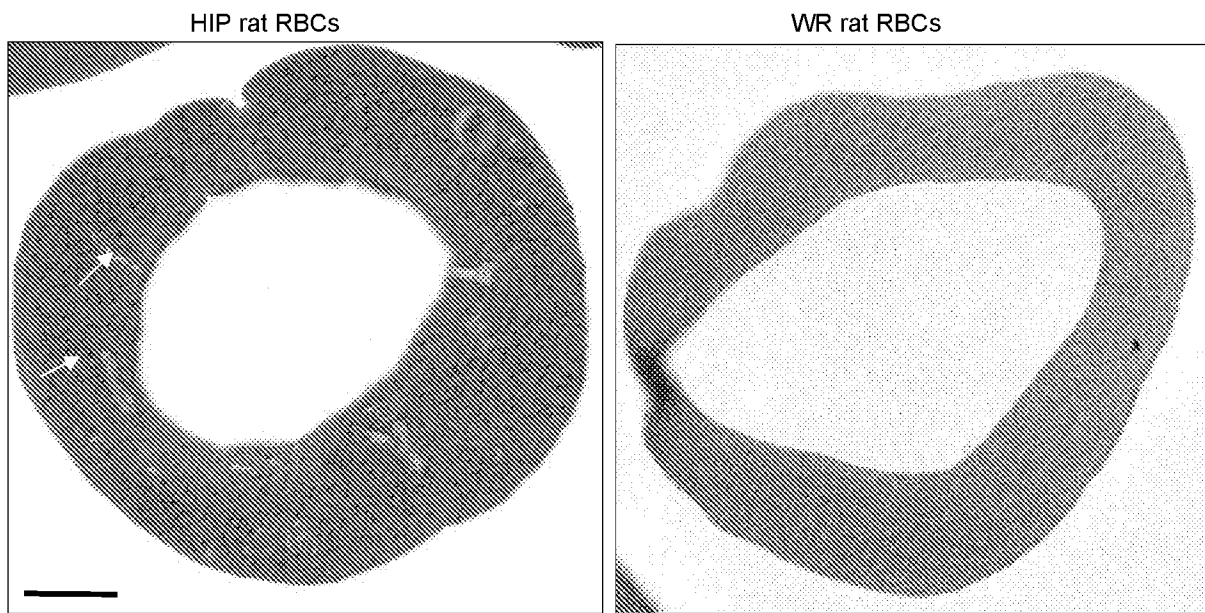


FIG. 26

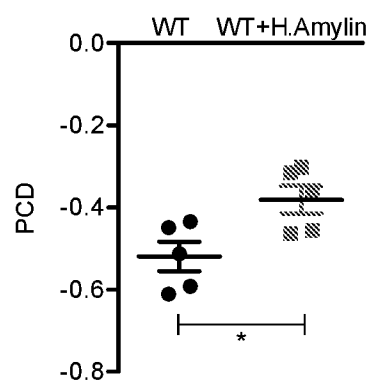


FIG. 27

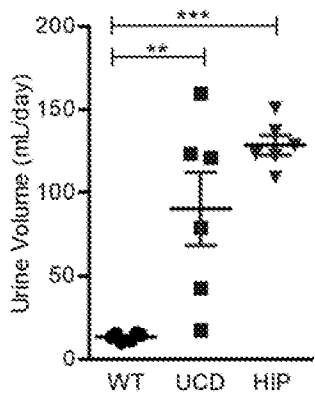


FIG. 28A

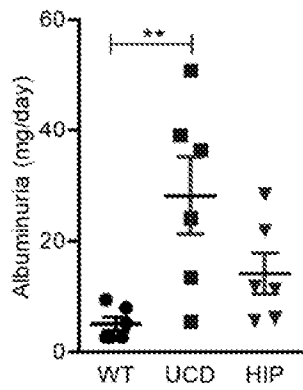


FIG. 28B

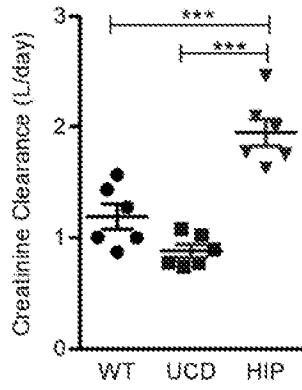


FIG. 28C

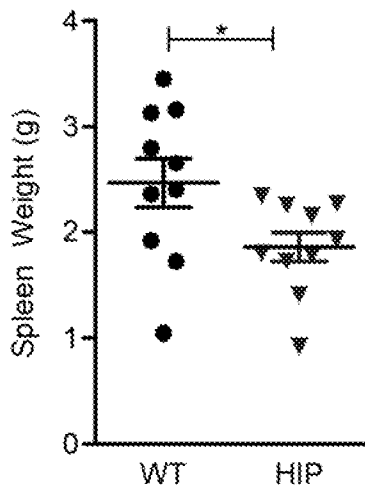


FIG. 29

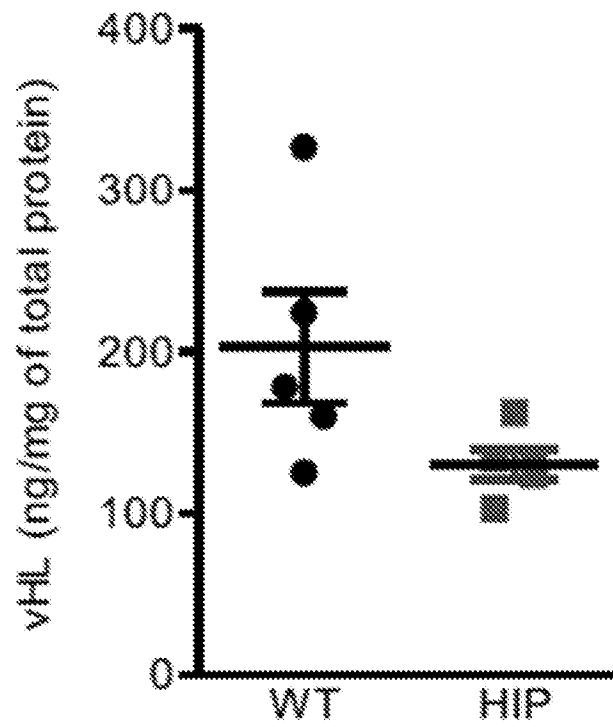


FIG. 30

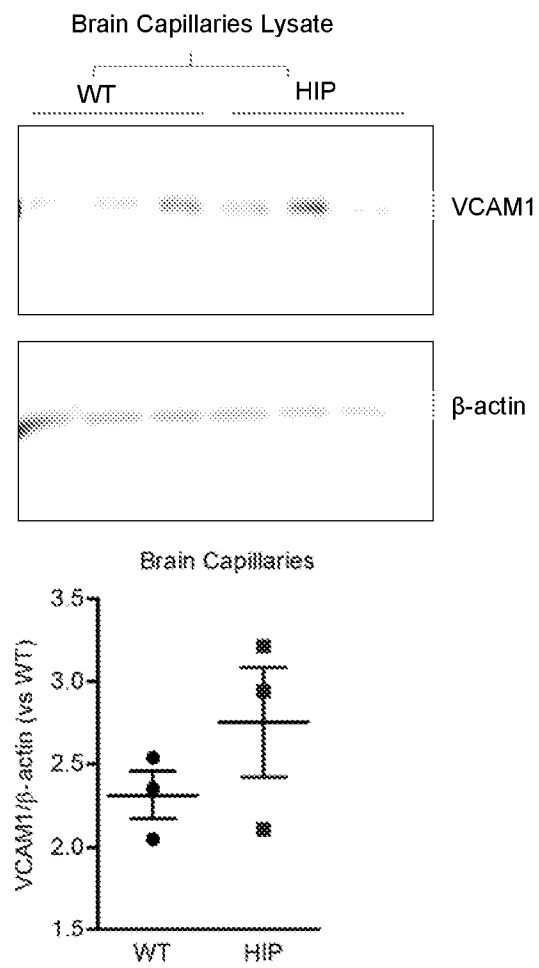


FIG. 31

FIG. 32A

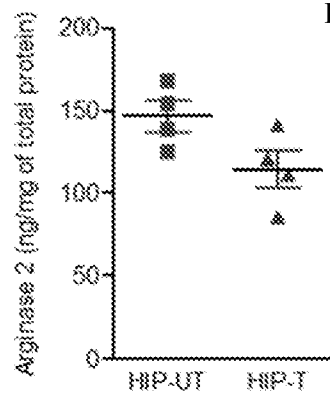
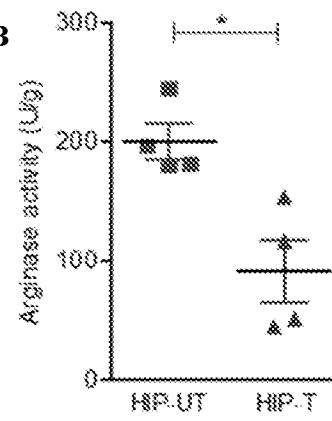


FIG. 32B



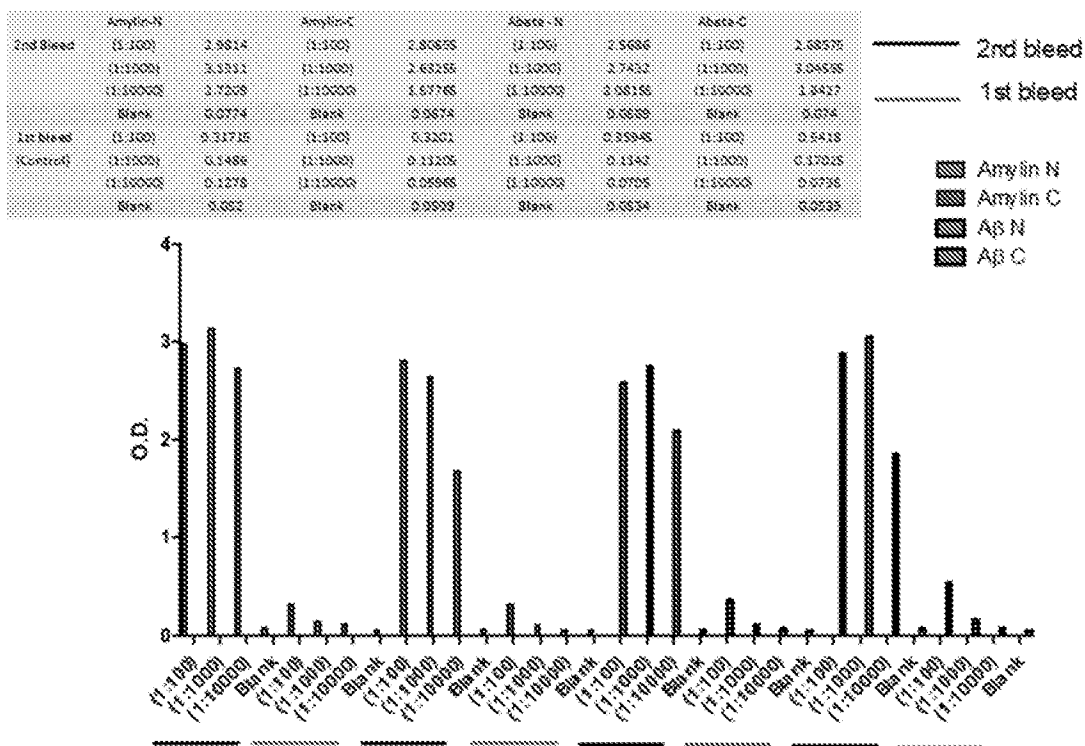
Titer of 1st and 2nd Bleed

FIG. 33 A

Titer of 2nd and 3rd Bleed

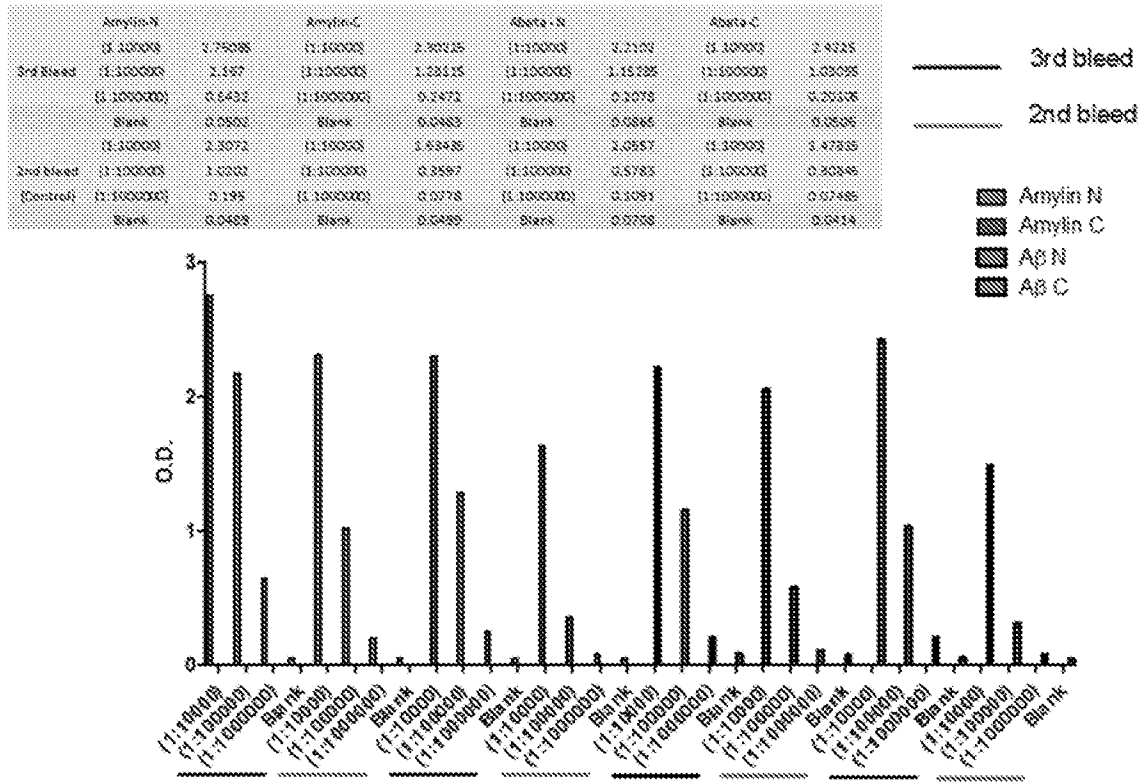


FIG. 33 B

Titer of 3rd and 4th Bleed

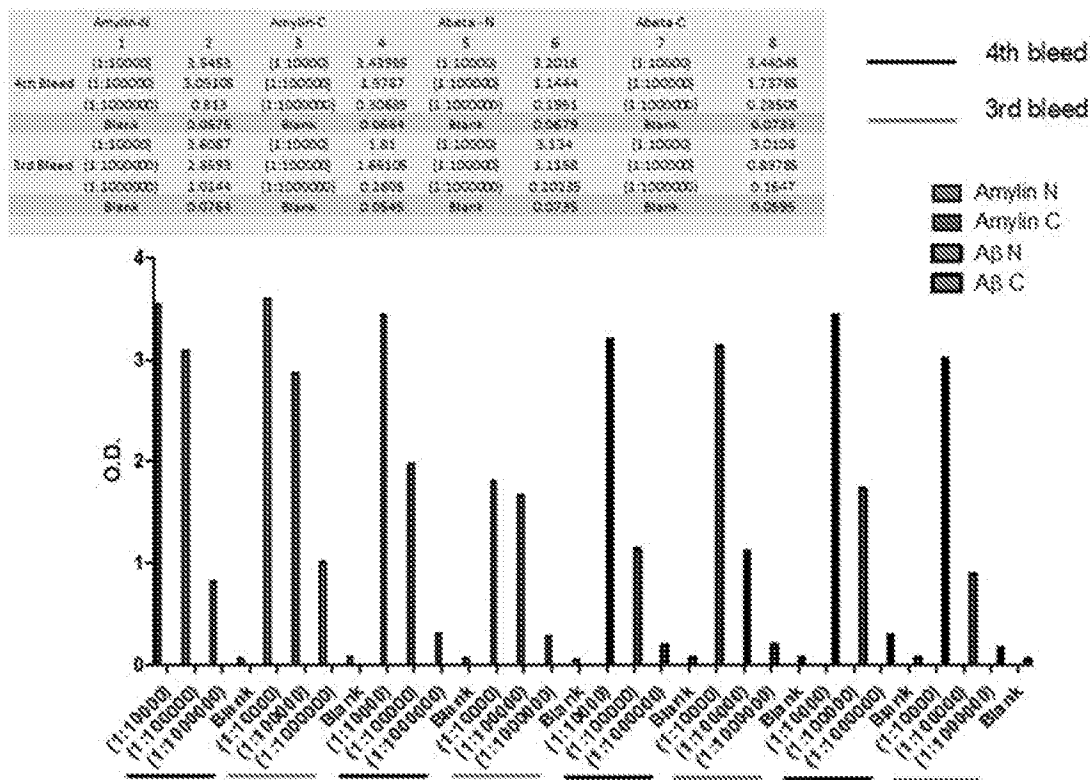


FIG. 33 C

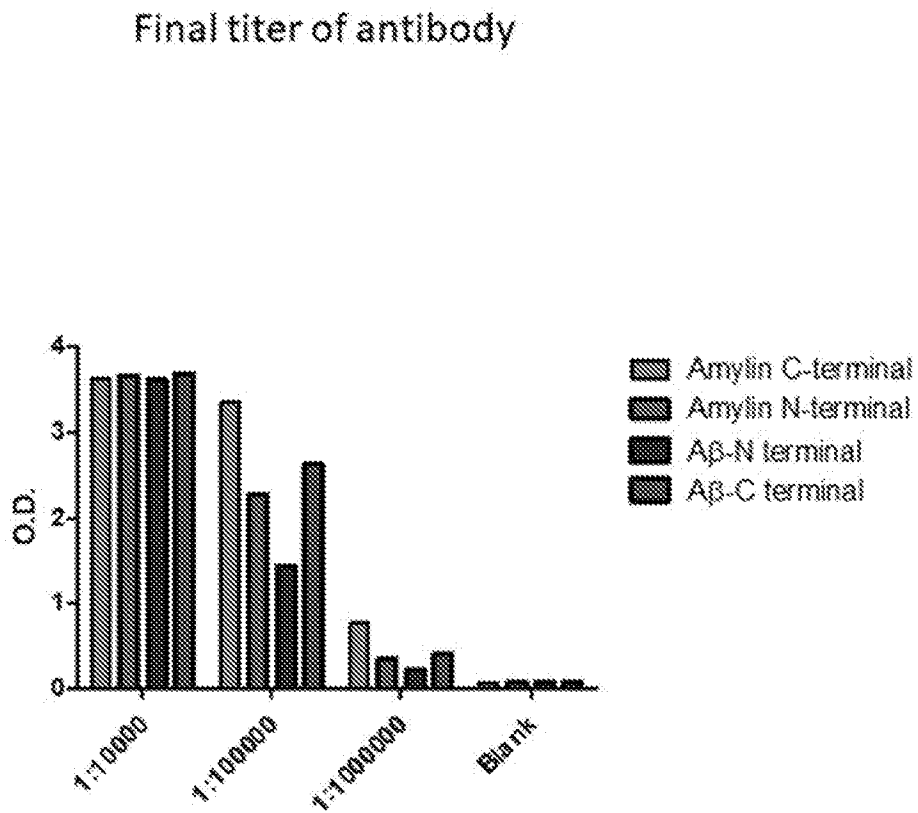


FIG. 33 D

FIG. 34A

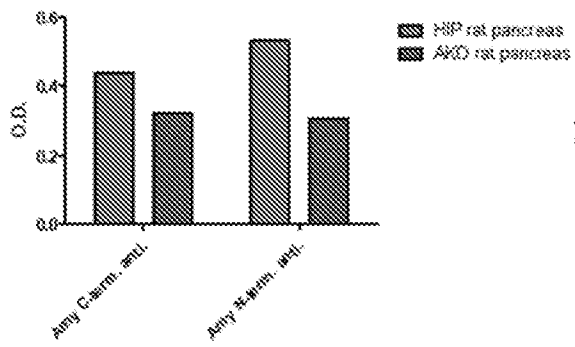


FIG. 34B

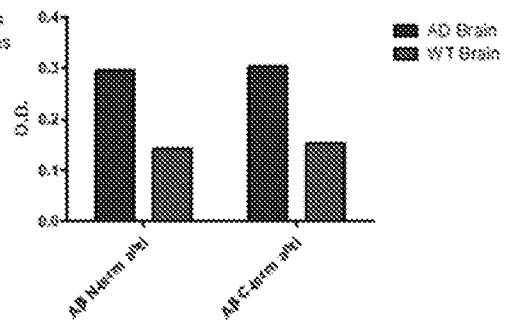
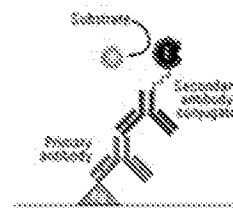


FIG. 34C



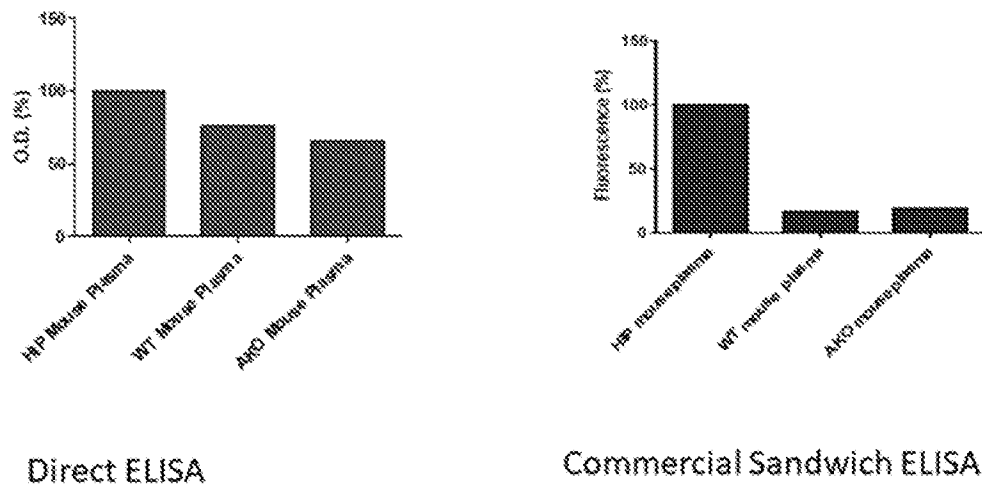


FIG. 35

FIG. 36A.

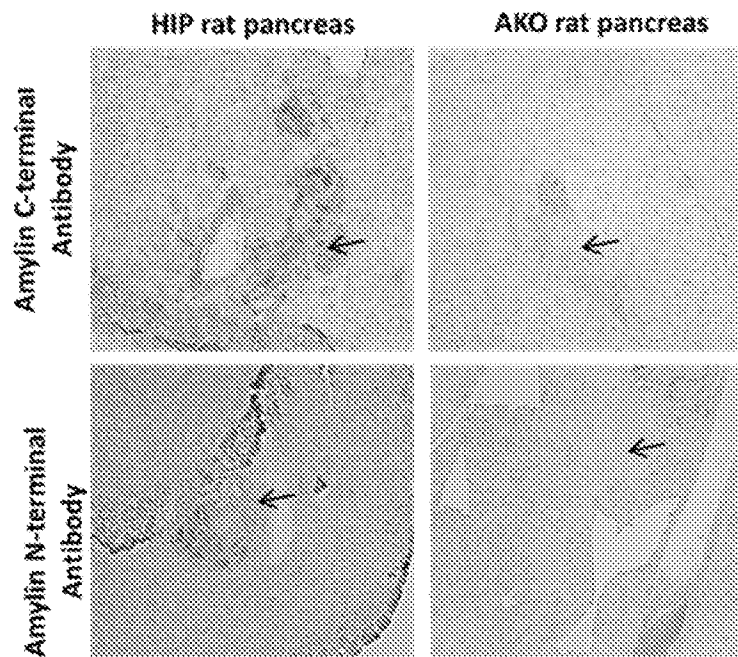
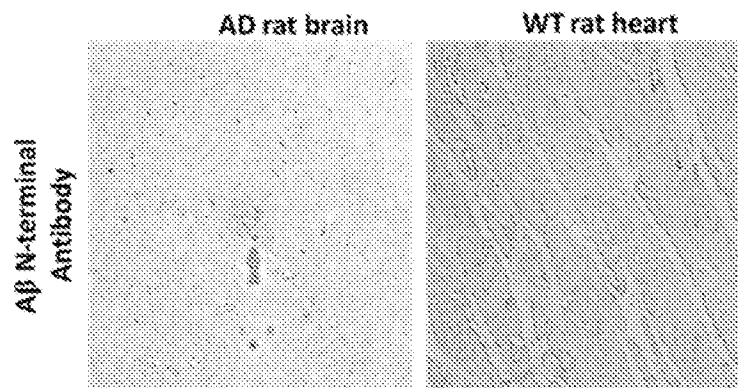


FIG. 36B.



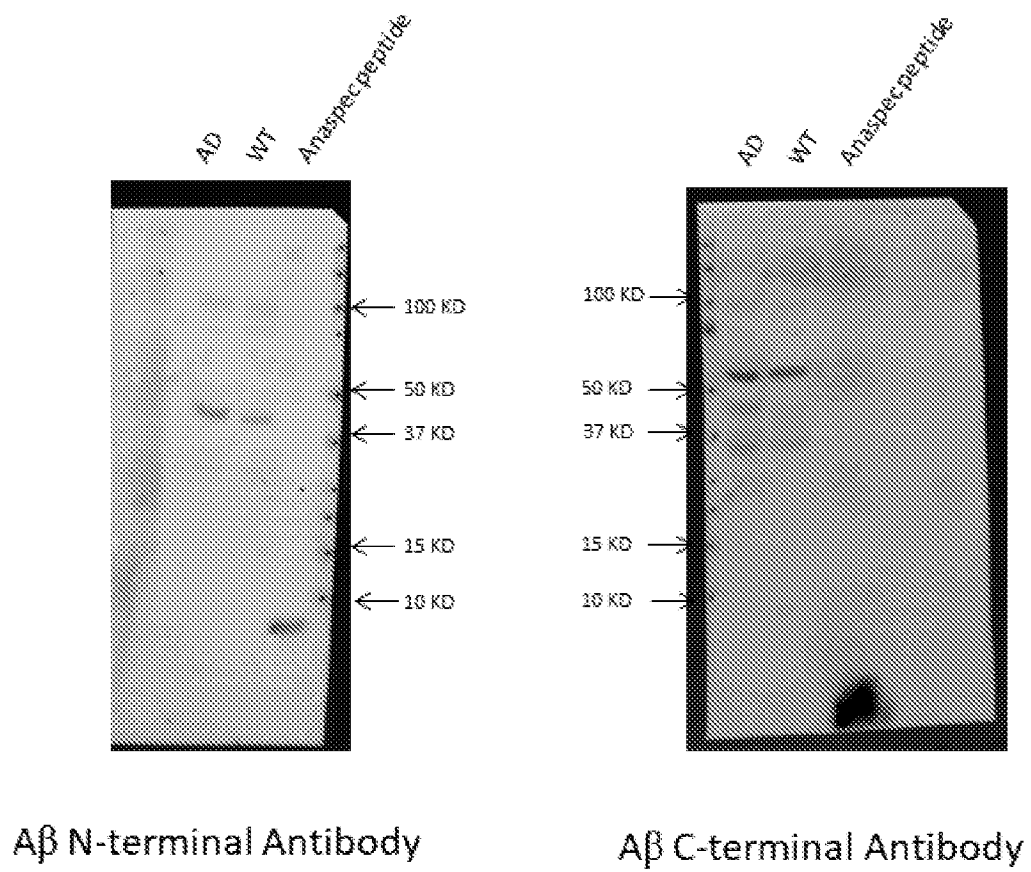


FIG. 37

FIG. 38A

Amylin peptide-

KCNTATCATQRLANFLVHSSNNFGAILSSTNVGSNTY

Immunogen-1 ~ 5'-CNNFGAILSSTNVGSN-3' (Amylin C)

Immunogen 2- 5'-CKCNTATCATQRLANFLVHSS-3' (Amylin N)

Amyloid β peptide-

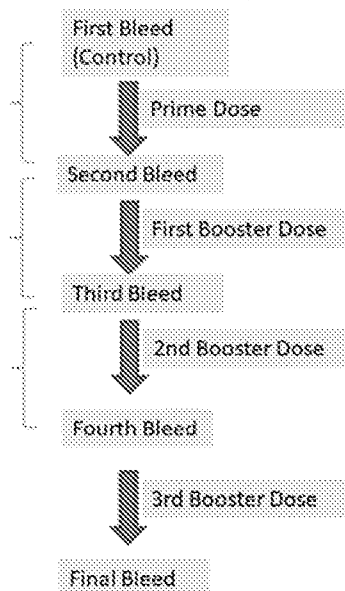
DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA

Immunogen-1 ~ 5'-CDAEFRHDSGYEVHHQ-3' (A β N)

Immunogen-2 ~ 5'-CKLVFFAEDVGSNKGAIIGLMVGGVVIA-3' (A β C)

FIG. 38B

Injection and Bleed Collection Schedule



INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2019/061518

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61B 5/00; C07K 16/18; C12N 15/02; C12P 21/08; G01N 33/53; G01N 33/68 (2020.01)

CPC - G01N 21/76; G01N 33/48714; G01N 33/5091; G01N 33/66; G01N 33/6893; G01N 33/74; G01N 2021/6439; G01N 2333/62; G01N 2333/65; G01N 2500/04; G01N 2800/04; G01N 2800/042; G01N 2800/325; G01N 2800/52; G01N 2800/56; G06Q 50/22 (2020.01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

USPC - 435/7.1; 435/7.21; 435/7.92; 514/6.9; 702/19 (keyword delimited)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	US 2009/0012716 A1 (URDEA et al) 08 January 2009 (08.01.2009) entire document	1, 3, 5 ----- 2, 19, 20
Y	DESPIA et al. "Cardioprotection by Controlling Hyperamylinemia in a 'Humanized' Diabetic Rat Model," Journal of the American Heart Association, 21 August 2014 (21.08.2014), Vol. 3, No. 4, Pgs. 1-15. entire document	2, 20
Y	US 2015/0210759 A1 (NEURIMMUNE HOLDING AG) 30 July 2015 (30.07.2015) entire document	19
P, X	VERMA et al. "Diabetic Microcirculatory Disturbances and Pathologic Erythropoiesis are Provoked by Deposition of Amyloid-Forming Amylin in Red Blood Cells and Capillaries," Kidney International, 05 September 2019 (05.09.2019), Vol. 97, Iss. 1, Pgs 143-155. entire document	1-20
P, X	US 2019/0331696 A1 (UNIVERSITY OF KENTUCKY RESEARCH FOUNDATION) 31 October 2019 (31.10.2019) entire document	1-20
A	US 2013/0143804 A1 (DESPIA et al) 06 June 2013 (06.06.2013) entire document	1-20
A	MANSOR et al. "Increased Oxidative Metabolism Following Hypoxia in the Type 2 Diabetic Heart, Despite Normal Hypoxia Signalling and Metabolic Adaptation," Journal of Physiology, 17 November 2015 (17.11.2015), Vol. 594, Iss. 2, Pgs. 307-320. entire document	1-20

☒ Further documents are listed in the continuation of Box C.☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

13 January 2020

Date of mailing of the international search report

11 FEB 2020

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2019/061518

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WONG et al. "Spontaneous Diabetes in Hemizygous Human Amylin Transgenic Mice That Developed Neither Islet Amyloid nor Peripheral Insulin Resistance," Diabetes, 15 July 2008 (15.07.2008), Vol. 57, Iss. 10, Pgs. 2737-2744. entire document	1-20

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2019/061518

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

a. ☒ forming part of the international application as filed:

☒ in the form of an Annex C/ST.25 text file.

☐ on paper or in the form of an image file.

b. ☐ furnished together with the international application under PCT Rule 13ter.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

c. ☐ furnished subsequent to the international filing date for the purposes of international search only:

☐ in the form of an Annex C/ST.25 text file (Rule 13ter.1(a)).

☐ on paper or in the form of an image file (Rule 13ter.1(b) and Administrative Instructions, Section 713).

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments: