Suggested journals:

1. Advances in Wound Care (4.9) Q1
2. Journal of Plastic and Reconstructive Surgery (3.6) Q1
3. International Wound Journal (3.1) Q1
4. Wound Repair and Regeneration (2.9) Q1
5. Journal of Plastic, Reconstructive & Aesthetic Surgery (2.7) Q1
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Prof. Gerry McElvaney	Royal College of Surgeons in Ireland, RCSI Education & Research Centre, Beaumont Hospital, Dublin, Ireland	gmcelvaney@rcsi.ie
Prof. Arthur Vandenbark	Neurology, School of Medicine, Oregon Health & Science University, Portland, OR, USA	vandenba@ohsu.edu
Dr. Amiq Gazdhar, PhD	Department of Pulmonary Medicine, University Hospital Bern, Switzerland	amiq.gazdhar@dkf.unibe.ch
Prof. Tanaya Shaw	Centre for Inflammation Biology and Cancer Immunology (CIBCI), King's college, UK	tanya.shaw@kcl.ac.uk
Prof. Herbert Tilg	Innsbruck Medical University, Innsbruck, Austria	herbert.tilg@i-med.ac.at

Role for Glycation of α1-Antitrypsin in the Pathophysiology of Impaired Diabetic Wound Healing: Mouse and Cell Culture Models

Farber Idan[1,2], Naumchik Alon[1], Istoyler Yosef[1], Zaknoun Melodie[1], Anav Yuval[1], Sheffer Lihie[1], Schuster Ronen[1], Halpern Dor[1,2], Lewis Eli C[1] and Silberstein Eldad[1,2]

[1]Department of Clinical Biochemistry and Pharmacology, Faculty of Health Sciences, Ben-Gurion University of the Negev, Beer-Sheba,84101, Israel, and [2]Plastic and Reconstructive Surgery Department, Soroka University Medical Center, Beer-Sheba,84101, Israel

Corresponding author: Idan Farber

Email: idani166@gmail.com

OrcID: https://orcid.org/0009-0004-6368-5374

FIGURES → PPT

Abstract

Impaired wound healing causes considerable morbidity among diabetic patients. Human α1-antitrypsin (hAAT) modulates inflammation within wound sites toward resolution. Upon glycation, gly-hAAT loses anti-proteolytic activity, but whether it fails to modulate inflammation and promote wound repair is unknown. **Objective:** Explore the impact of clinical-grade hAAT on wound repair under hyperglycemic conditions, and the role of gly-hAAT in impaired wound healing pathophysiology. Research **Design and Methods:** Mice were rendered hyperglycemic and excisional wounding was performed, treated with topical albumin or hAAT from time of wounding and every three days. Wound area was followed and samples collected for histology and gene expression analysis. Gly-hAAT was generated from clinical-grade hAAT in laboratory settings. In-vitro, RAW 264.7 macrophage responses were assessed and re-epithelialization was tested using A549 cells in the presence of gly-hAAT, and in the presence of sera from individuals with poor glucose control, both supplememnted with clinical-grade hAAT. **Results:** Topical hAAT accelerated in-vivo and in-vitro wound closure. Vascular maturity appeared earlier in hAAT-rich conditions, and gene expression skewed towards anti-inflammatory IL-1β/IL-1Ra ratio. Gly-hAAT inhibited normoglycemic mouse wound closure and epithelial cell gap closure, both systems rescued by clinical-grade hAAT. Gly-hAAT evoked an inflammatory response in macrophages, and diabetic patient serum inhibited epithelial cell gap closure; both trends were reversed by clinical-grade hAAT. **Conclusions:** Topical hAAT accelerates wound closure under hyperglycemic conditions, and gly-hAAT is inflammatory and fails to benefit wound repair. Considering its phenomenal safety profile, it is suggested that clinical-grade hAAT is primed for testing in clinical settings.

Introduction

The most important role of the skin is to create a barrier between the outside and internal environments, protecting the organism from external hazards and preventing excessive loss of fluid. Therefore, in cases of skin injuries, it is extremely important to maintain skin integrity in a rapid and efficient manner. The wound healing process involves three partially overlapping stages: inflammation, proliferation, and remodeling, each holding a typical cellular and molecular profile (1–3). An aberrant orchestration of these stages might occur when local inflammatory triggers persist, be it in the form of local infection, residual inflammatory agents, ischemia or mechanical disruption, resulting in difficult-to-heal wounds. Persistent hyperglycemia is particularly known for setting the grounds for difficult-to-heal wounds (3); as such, it poses a significant risk for complications, such as lower limb amputations (4,5). Wounds that develop in individuals with poor glycemic control depict a predominance of neutrophils, as well as M1 macrophages that typically express inducible nitric oxide synthase (iNOS), representative of the inflammatory stage, alongside inadequate revascularization (6–8) and might also be accompanied by a bacterial biofilm (9). Perhaps more than other etiologies of difficult-to-heal wounds, this environment is also characterized by disruption in the functions of fibroblasts, endothelial cells and epithelial cells (5,10).

The instigation of an appropriate inflammatory response is characterized by a surge in pro-inflammatory cytokine production, such as IL-1β, TNFα and IL-6 (4,11), and the appropriate migration of neutrophils and activated macrophages for elimination of pathogens and removal of injured cell debris (2); the inflammatory environment is also necessary for activating local fibroblasts, the process of re-epithelialization, angiogenesis and revascularization, and the advancement of immune recruitment (5,10,12). However, the inflammatory response should ideally be efficient enough also to elicit a gradual conversion to inflammatory *resolution*; excessive inflammation, therefore, will interfere with the desired unfolding of the two latter stages of wound healing, i.e., proliferation and remodeling. The molecular and cellular profile of an inflammatory resolution site includes M2-like macrophages that typically express arginase-1 (Arg-1) and CD206 (13), and the proactive expression of anti-inflammatory cytokines, such as IL-1 receptor antagonist (IL-1Ra) (14). IL-1Ra mitigates upstream IL-1-elicited inflammatory processes by preventing inflammatory

IL-1 family members from binding to IL-1 receptor, blocking the release of yet more IL-1β (15). By this, the balance between IL-1β and IL-1Ra holds immense significance in wound repair outcomes (16).

Human α1-antitrypsin (hAAT) is a 52 kDa circulating glycoprotein that belongs to the serine protease inhibitor (SERPIN) family, as reviewed elsewhere (17). Its circulating levels are dependent on liver production, although mucosal tissues produce local hAAT, and some other cell types have been observed to produce hAAT. hAAT inhibits inflammatory serine-proteases, such as neutrophil elastase, which would otherwise cleave and activate stimulatory protease-activated receptors (PARs) on the surface of immunocytes, and also cleave and activate locally deposited cytokine precursors, such as pro–IL-1β released from dying cells (18–23). Its circulating levels are dependent on liver production of hAAT, although mucosal tissues produce local hAAT and some other cell types have been observed to produce hAAT. hAAT inhibits inflammatory serine-proteases, such as neutrophil elastase, which would otherwise cleave and activate stimulatory protease-activated receptors (PARs) on the surface of immunocytes, and also cleave and activate locally deposited cytokine precursors, such as pro-IL-1β (17). It is not surprising, therefore, that lung alveolar wall degradation represents the hallmark finding in patients with genetic AAT deficiency (AATD) (24–26).

Although less common, AATD patients present dermatological manifestations, such as panniculitis, vasculitis, psoriasis, urticaria, and angioedema(27), and suffer from overall impaired wound healing (28). Yet, while inflammation is indeed diminished in hAAT-rich conditions, hAAT is appreciated as an immune modulator that directly affects neutrophils, macrophages, dendritic cells and B lymphocytes, and in an indirect manner T cells and NK cells (29–36). AAT also harbors resolution-promoting properties independent of its anti-protease activity, suggesting that some of its activities are mediated by surface binding interactions with local agents (37). For example, hAAT increases the production of inflammation-induced IL-1Ra in a manner independent of protease inhibition (37,38), and binds to IL-8 and to several danger-associated molecular pattern molecules (DAMPs) (39–41). Upon binding to local nitric oxide, hAAT turns anti-bacterial and effectively diminishes bacterial burden (29,31,42,43). Indeed, hAAT was found to promote wound healing processes at multiple levels (44–49), including improving the formation of mature

blood vessels (50,51) and decreasing the amount of necrotic tissue following ischemia (52), as well as accelerating epithelial gap closure in-vitro (37,53) and in in-vivo acute wound models in mice (32,53). Interestingly, the blockade of inflammation by corticosteroids *interferes* with the desired outcomes generated by hAAT, adding to the appreciation that hAAT is an inflammation-driven pro-resolution agent (37).

Protein glycation is a non-enzymatic covalent attachment of glucose to lysine and arginine residues of a protein, yielding advanced glycation end-products (AGEs) (54,55). Glycated hemoglobin (HbA1c) is a quintessential example, and is indeed used as an indicator for diabetes control; it also signifies the fact that de-glycation is enzymatic, and occurs in cells and not in the circulation (56). AGEs are inflammatory; they induce IL-1β, IL-6, and TNFα production, promote oxidative stress, and impair wound healing (3,4,57,58). hAAT undergoes direct glycation under hyperglycemic conditions, mostly in the liver, losing its ability to inhibit serine proteases (59,60); however, the effect of glycation on its ability to control inflammation has yet to be tested.

The present study hypothesizes that hAAT might fail to protect tissues from injury when it is glycated, both by the lack of ability to act as a pro-resolution agent, and as a gain of function phenomenon by which glycated hAAT becomes an inflammatory agent.

Materials and Methods

Animals

Animal studies were approved by the Institutional Animal Care and Use Committee (Approval BGU302-1-2024D) and conducted in line with the Guide for the Care and Use of Laboratory Animals, 8th Edition. C57BL/6 mice (8-12-week–old females, Envigo+ Laboratories, Inc., Rehovot, Israel) were housed at a standard viva Mice transgenic for human AAT (hAAT$^{+/+}$) on a C57BL/6 background were generated at the University of British Columbia, Canada (61). hAAT$^{+/+}$ mice express constitutive levels of hAAT in their blood (<1 μg/ml) and as a result, they are frequently used as a positive control for AAT treatment.

Induction of hyperglycemia in mice

Wild-type (WT) mice were weighed prior to any interventions. Hyperglycemia was induced using streptozotocin (STZ) purchased from Sigma Aldrich, Rehovot, Israel. STZ was prepared at a concentration of 150mg/kg in a buffer solution composed of 50 mM sodium citrate, adjusted to pH 4.5. Mice received a single intraperitoneal injection of the STZ solution at a volume of 250µl per mouse.

Following STZ administration, blood glucose levels were measured daily to monitor the onset of hyperglycemia. Hyperglycemia was defined as a non-fasting blood glucose level of 250mg/dl or higher, or a fasting blood glucose level of 120mg/dl or above. The mice were observed for a week post-STZ injection to ensure the stable establishment of hyperglycemia.

In vivo wound healing models

One week into hyperglycemia, mice were anesthetized using isoflurane inhalation (2.5% for induction, 2% for maintenance). Post-anesthesia, the dorsal hair was removed with an electric razor, and the skin was disinfected with chlorhexidine gluconate (0.5% w/v) in a 70% v/v ethanol solution. An 8mm surgical excision was then made on the disinfected dorsal side. Following the incision, mice were categorized into three treatment groups: a hyperglycemic control receiving albumin, a hyperglycemic group treated with hAAT (Glassia, Kamada LTD., Nes-Ziona, Israel), and a normoglycemic control. Treatments, formulated at 4 mg/kg, were administered in a 100µl volume directly to the wound bed, beginning on the day of the incision and then every three days. The healing process was tracked by photographing the wounds every three days, with subsequent image analysis and measurements conducted using the ImageJ software (MedCalc Software, Ostend, Belgium).

Histological Analysis

Animals were sacrificed, wounds were excised and immediately immersed in 10% neutral-buffered formalin (Sigma-Aldrich, Rehovot, Israel). Samples were then placed facing the microtome blade for paraffin embedding. Tissues were

subsequently cut into 4-6µm sections, mounted on slides, and stained with Hematoxylin and Eosin (H&E; Jackson Immuno Research, West Grove, PA, USA). Identification of leukocytes was undertaken as described elsewhere (62).

Glycation of hAAT in acellular conditions

Non-enzymatic glycation of hAAT was conducted to evaluate the implications of glycosylation on its inhibitory features, adapting the methodology described by Duell et al. (63,64). For the glycation process, a 0.5 ml sample of hAAT (equivalent to 0.59 mg) in 2 ml of 50 mM Tris-HCl buffer (pH 8.0) was combined with 12 mg/ml sodium borohydride. D-glucose was then added at concentrations of 400, 500, 800 and 4000 mM to individual vials. These preparations were sealed under nitrogen and incubated at 37°C for 7 days. Post-incubation, the glycosylated solutions underwent extensive dialysis against the initiating buffers to purge unreacted glucose and sodium borohydride. The extent of glycation in the resulting gly-hAAT was ascertained via assays using either picryl sulfonic acid or trinitrobenzene sulfonic acid (TNBS) (64).

Western blot analysis

Protein concentrations in the samples were determined using the BCA protein assay kit (Cat#202389, Santa Cruz Biotechnology). For each sample, 20µg of protein were loaded and resolved on 7.5-18% SDS-PAGE. This electrophoresis was conducted to estimate the size differences between the native hAAT and its various glycated forms (gly-hAAT) at glycation levels of 400, 500, 800 and 4000. The proteins were subsequently electro-transferred onto nitrocellulose membranes (Cat#1620147, Bio-Rad, CA, USA). Following transfer, membranes were blocked with a 5% BSA prepared in Tris-Buffered Saline with 0.1% Tween 20 (TBS/T) for 45 minutes at room temperature. The membranes were then probed with a primary antibody specifically recognizing hAAT (Mouse anti-Human AAT antibody, 1/1000, Cat#VMA00662, Bio-Rad, CA, USA) followed by detection with goat anti-mouse HRP-conjugated antibody (1/10,000, STAR207P). The protein-antibody complexes were visualized using the enhanced chemiluminescence (ECL) method (Cat#XLS063, Cyanagen, Bologna, Italy).

Elastase activity assay

Neutrophil elastase activity was determined in acellular conditions using a designated kit (Sigma-Aldrich, St. Louis, MO, USA), according to the manufacturer's instruction. Briefly, hAAT/Gly-hAAT were incubated at indicated concentrations with elastase (0.39 μM) and the kinetics of color product determined. Enzymatic activity is represented as percent from maximal activity.

In vitro A549 epithelial gap repair assay

In vitro scratch assay was performed using A549 cells. Cells were grown to confluence in 24-well plates and uniform wounds were inflicted using a sterile 200 μl pipette tip, thus creating a cell-free area, as described elsewhere (37,53). Cultures were washed twice with complete RPMI 1640 supplemented with 2.5% FCS (both from Biological Industries Inc., Beit Haemek, Israel). Treatments were introduced directly onto cells in 2.5% FCS; control conditions included 2.5% media. Images were acquired immediately after wounding and then again 6, 12, 24, and 36 hours later using a photomicroscope (Zeiss). Images were analyzed by ImageJ, and cell-free areas were marked; outcomes are represented as percent from the initial wound area.

Macrophage culture

RAW 264.7 cells (Cat#SC-6003, ATCC) were seeded in 48-well plates (1×105 cells/well) in RPMI 1640 medium supplemented with 5% FCS (Biological Industries). Cells were incubated for 4 hours with indicated concentrations of hAAT or Gly-hAAT and then stimulated with 5 ng/ml LPS. Cell culture supernatants were collected after 8 hours. Levels of mouse TNFα were determined by specific ELISA (Mouse TNF-alpha DuoSet ELISA, Cat#DY410, R&D systems, Minneapolis, USA), All measurements were performed in triplicate.

RT-PCR gene expression analysis

RNA was extracted from mouse wound samples for gene expression analysis. Briefly, tissue was transferred to a polytron homogenizer, and the homogenized samples were loaded into 1.5 ml RNase-free microcentrifuge tubes. RNA was extracted using Gynzol® reagent (Invitrogen, Waltham, MA, USA) and isolated on columns (RNAqueous®-Micro Total RNA Isolation Kit, Thermo Fisher Scientific, MA, USA), following manufacturer's guidelines. Eluted RNA was then quantified using NanoDrop (Wilmington, DL, USA), and 200 ng of RNA were reverse transcribed into cDNA using Prime Script RT Reagent kit (Quanta Biotech, USA). Quantitative PCR was performed using an RT-PCR system (StepOnePlus™ Real-Time PCR System, ThermoFisher Scientific Corporation, Waltham, MA, USA), and SYBR Premix Ex Taq II (Quanta Biotech, USA) at a 20µl volume reaction. CFX96 manager software was used to determine threshold cycle values; beta actin was used as reference gene. Primers were designed for murine transcripts, as follows: IL-1β '5-CTT CCA GGA TGA GGA CAT GAA GG-3′ (forward), '5-AGT GCA GTT GTC TAA TGG GA-3′ (reverse); VEGF, 5′-TGG GAC TGG ATT CGC CAT TT-3′ (forward), 5′-GTG GGT GGG TGT GTC TAC AG-3′ (reverse); IL-1Ra, 5′-GAC CCT GCA AGA TGC AAG CC-3′ (forward), 5′-GAG CGG ATG AAG GTA AAG CG-3′ (reverse); MCP-1 5′-AGG CAT CAC AGT CCG AGT CA-3′ (forward), '5-CCA CAA CCA CCT CAA GCA CT-3′ (reverse), ARG1 5′-AAC ACG GCA GTG GCT TTA ACC-3′ (forward), '5-GGT TTT CAT GTG GCG CAT TC′ (reverse), CD206 5′-GGC TGA TTA CGA GCA GTG GA-3′ (forward), '5-CAT CAC TCC AGG TGA ACC CC-3′ (reverse), CD14 5′-CAG AGA ACA CCA CCG CTG TA-3′ (forward), '5-ACA CGC TCC ATG GTC GGT A-3′ (reverse) and ACTB 5′-CAT TGC TGA CAG GAT GCA GA-3′ (forward), '5-TGC TGG AAG GTG GAC AGT GA-3′ (reverse).

Human sample collection and processing

The study was approved by the Institutional Review Board of Soroka University Medical Center (SOR 1106-2018), ensuring adherence to ethical guidelines for biomedical research. Nine adult patients diagnosed with diabetes were recruited by the diabetes clinic at Soroka University Medical Center, Israel, for serum sampling. The diagnosis was confirmed based on established criteria, including glycated hemoglobin (HbA1C) levels and random fasting blood glucose measurements. Prior to sample collection, informed consent was obtained from all participants

Statistical Analysis

All quantitative data were presented as mean±SEM. The statistical significance of the differences between groups was evaluated using ANOVA followed by post-hoc tests for multiple comparisons. A p-value <0.05 was considered statistically significant. Statistical processing was performed using GraphPad Prism software (GraphPad Software, La Jolla, CA, USA).

Results

Circulating transgenic hAAT accelerates wound closure in hyperglycemic mice

The impact of hAAT therapy on the aberrant wound healing process that arises from hyperglycemia was addressed using a mouse model for hyperglycemia. Mice homozygous for hAAT were compared to WT mice. As shown in Fig. 1A, the degree of hyperglycemia was comparable between groups. Upon developing hyperglycemia, groups were compared for their capacity to repair an excisional wound (**Fig. 1B**). As shown, normoglycemic WT mice (CT) began gradually closing their wounds after day 4 from wounding (wound area day 4, 81.50±38.91 and day 6 38.00±11.14, percent from day 0, mean±SEM, $p<0.05$ between wound areas at both time points); in contrast, hyperglycemic WT mice (WT) were unable to close their wounds for the first 8 days, afterwhich follow-up was ceased due to ethical concerns. Mice that were hyperglycemic and had elevated circulating hAAT ($hAAT^{+/+}$) appeared to initiate closure between days 2 and 4, albeit without reaching statistical significance compared to wound area in hyperglycemic WT mice at those time points. Nonetheless, the two hyperglycemic groups significantly diverged from one another on days 6 and 8 from wounding, where a clear superior capacity for wound closure was evident in hAAT mice (wound area day 6, 106.30±36.57 and 62.75±12.50, mean±SEM, $p<0.05$ between WT and $hAAT^{+/+}$ wound, and day 8 (108.5±23.63 and 48.25±18.52, mean±SEM, $p<0.05$ between WT and $hAAT^{+/+}$ wound areas at both time points).

Topical clinical-grade AAT accelerates wound closure in hyperglycemic mice

Since circulating hAAT achieved improved wound repair in hyperglycemic mice, the possibility of local treatment with hAAT in hyperglycemic mice was tested (Fig. 2). For this purpose, the control group consisted of local topical human albumin treatment at the same dose and timing as clinical grade hAAT. The excisional wound performed was 8mm instead of 5mm, and a lower dose of STZ was applied so as to better reflect poorly-controlled blood glucose levels in patients. As shown in **Fig. 2A**, hyperglycemia was comparable between the groups. According to follow-up of wound area percentages that are summarized in **Fig. 2B**, the albumin-treated, hyperglycemic mouse group exhibited poor wound closure copmere to

normoglycemic mice, whereas hyperglycemic mice receiving hAAT treatment demonstrated a significantly more robust healing response compared to albumin-treated wounds. In order to better appreciate the differences between groups, a day-to-day analysis is presented in **Fig. 2C**. As shown, hyperglycemia significantly impeded wound closure, starting on days 3 (30.35±6.36 vs. 43.31±19.35, normoglycemic and albumin-treated hyperglycemic mice, p=0.0153). However, hyperglycemic mice receiving hAAT treatment exhibited more substantial wound closure than their albumin-treated counterparts (38.23±13.78). By Day 6, this trend persisted. Control normoglycemic mice (CT) displayed a mean wound closure rate of 22.80±5.22, contrasting with a significantly higher percentage of residual wound 30.97±9.86 in hyperglycemic mice treated with albumin (p=0.022). hAAT-treated mice demonstrated superior wound closure of 17.55±7.98 (p<0.001). On day 9, although hyperglycemic mice continued to lag behind in wound closure, and hAAT treatment still conferred benefits, the differences were not statistically significant.

Histological profile of wounds in hyperglycemic mice treated with topical clinical-grade AAT

To undertake an additional assessment of the wound's condition after hAAT-treatment, day-3 wound samples were collected for histological analysis. As shown in **Figure 3,** a wound can be divided into three distinct areas: edge zones, inflammatory zones, and granulation tissue. The hyperglycemic control group (**Fig. 3A**) exhibited infiltrates abundant in immune cells, characterized by extensive nuclear debris and substantial netosis. Inside the granulation site there are filtered red blood cells without concomitant formation of mature blood vessels. In contrast, wounds treated with hAAT (**Fig. 3B**) displayed a structured organization of the tissue, characterized by regions of immune filtration and reduced nuclear debris and netosis. The presence of mature blood vessels carrying red blood cells is noted.

Gene expression analysis of wounds in hyperglycemic mice treated with topical clinical-grade AAT

In order to explore the changes that underlie the enhanced rate and superior histological findings of wounds in hyperglycemic mice treated with topical hAAT,

wound tissue was sampled at day 3 from wounding for mRNA transcript analysis of key agents involved in wound healing. As shown in **Figure 4A**, compared to wounds from normoglycemic mice, wounds from hyperglycemic mice treated with albumin (control) exhibited elevated transcript levels of VEGF and IL-1β. Mice treated with hAAT displayed VEGF transcript levels that were closer to normoglycemic mice, albeit without reaching statistical significance. IL-1β transcript levels were higher in both hyperglycemic groups compared to normoglycemic mice. Unlike the changes observed in VEGF and IL-1β expression levels, IL-1Ra expression was significantly upregulated in wounds treated with hAAT. Since IL-1 receptor senses the ratio between IL-1β and IL-1Ra, a calculated ratio per animal is also presented; as shown, IL-1β/IL-1Ra ratio in wounds from hyperglycemic mice exhibits a predominance towards IL-1β. However, wounds from hyperglycemic mice treated with hAAT exhibited an intermediate decline in IL-1β and IL-1Ra ratio (CT: 0.010±0.002, STZ-ALB: 0.003±0.0007 and STZ-hAAT: 0.005±0.0001, arbitrary units).

Analysis of macrophage-related gene expression profile is presented in **Figure 4B**. As shown, wounds from hyperglycemic mice exhibited greater levels of CD14 transcripts and of MCP-1 transcripts, without reaching statistical significance. Wounds from mice treated with hAAT displayed little changes from wounds of hyperglycemic mice, both for CD14 and MCP-1 expression levels. Transcript levels of CD206 and Arg-1 were assessed and are normalized CD14 transcripts carried by macrophages. As shown, on day 3 from wounding, wounds from hyperglycemic mice were predominantly low in regards to CD206 and Arg-1 transcript levels, and treatment with hAAT did not alter this profile on day 3.

Glycated hAAT (gly-hAAT) displays inflammatory attributes and fails to inhibit LPS-induced TNFα release from macrophages

A glycated form, gly-hAAT, was generated in order to examine hyperglycemia-induced modifications in hAAT. As shown in **Figure 5A**, glycation of hAAT by exposure to glucose resulted in an increase in molecular weight (kD) in a glucose-concentration–dependent manner. The 800 mM glucose concentration was chosen for further investigation and hereby represents gly-hAAT. Apart from change in size, gly-hAAT was also affected at a functional level (**Fig. 5B**). According to an elastase activity assay, naïve hAAT effectively neutralized elastase activity at a 1:1

molar ratio, and inhibition by gly-hAAT required three-fold inhibitor concentrations (72.86±11.00%, 28.06±2.15% and 4.74±3.06% elastase activity, in the presence of ×1, ×2 and ×3 gly-hAAT, respectively).

Assessment of the inflammatory response of the RAW 264.7 macrophage cell line in the presence of gly-hAAT, is presented in **Figure 5C**. Glucose alone was introduced to cells to represent the effect of possible carryover of residual glucose molecules left after hAAT glycation protocol. As shown, steady-state cells released comparable levels of TNFα in the presence of control medium, hAAT, and glucose. Unexpectedly, cells exposed to gly-hAAT were induced to release significantly higher concentrations of TNFα (38.91±7.30 pg/ml, 429.30±247.6 pg/ml, control and gly-hAAT, respectively, $p<0.0001$). Administration of naïve hAAT together with gly-hAAT significantly reduced TNFα release, to levels comparable to whose obtained by exposure of cells to gly-hAAT alone (147.70±33.86 pg/ml, $p=0.018$).

Under LPS-stimulated conditions, TNFα was released ten-fold both in the presence of the control medium and hAAT. Glucose reduced TNFα release under LPS stimulation, albeit without reaching statistical significance, while gly-hAAT caused an increase in TNFα release, greater than the levels induced by LPS alone (3405.00±1010.00 pg/ml, 856.00±46.40 pg/ml, gly-hAAT/LPS and LPS, $p=0.009$).

Gly-hAAT interferes with epithelial gap closure in vitro

Following assessment of the inflammatory activity contained within gly-hAAT, its impact on epithelial gap closure was investigated (**Fig. 6**). According to the A549 epithelial cell line scratch assay, under control conditions, gap area was reduced to 39.41±8.50% in 24 hours. In the presence of hAAT, gap area was significantly smaller than in control conditions (29.32±9.80% gap, $p=0.040$). Glucose treatment resulted in a residual gap area of 25.14±4.96% at 24 hours ($p=0.015$ compared to control). In contraast, gly-hAAT–treated cells depicted gap closure that closely resembles control conditions (40.04±10.52% gap compared to hAAT-treated group at 24 hours, $p=0.070$).

Gly-hAAT interferes with wound closure in normoglycemic mice

Extending on the results obtained in-vitro, the effect of gly-hAAT on wound healing in-vivo was investigated (**Fig. 7**). Excisional wounds were performed in *normoglycemic* mice, and the possibility that a glycated form of hAAT interferes with spontaneous wound closure was directly examined. As shown in **Figure 7A**, while ALB-treated wounds exhibited a closure rate in which day-3 wound area was 21.35±5.38% from initial wounding, gly-hAAT–treated wounds were significantly slower to close and on that day, reached 37.00±4.58% area from initial wounding (mean±SEM, $p<0.001$). A day-to-day depiction of the differences between groups is presented in **Figure 7B**. As shown, day-6 outcomes were similar to outcomes from day 3. The apparent negative impact of gly-hAAT on wound closure rates was challenged by co-treatment with naïve hAAT; according to outcomes, naïve hAAT significantly accelerated wound closure despite the presence of gly-hAAT, resulting in a profile that is similar to albumin-treated wounds. For example, on day 3, the combination gly-hAAT/hAAT reached 19.64±5.06 area from initial wounding. On day-6, albumin-treated wounds reached 21.26±7.92 area from initial wounding, and gly-hAAT/hAAT reached 18.80±7.16 area from initial wounding. In addition, between days 3 and 6, gly-hAAT was still showing progressive closure (from 37.00±4.58 to 32.11±4.31) while gly-hAAT/hAAT had already plateaued.

The negative impact of sera from diabetic patients with poorly-controlled blood glucose on epithelial gap closure is attenuated by added clinical grade hAAT

Sera were collected from a cohort of diabetic patients with poorly-controlled blood glucose (N=10), and then directly introduced onto epithelial cells in an epithelial gap closure assay **(Fig. 8)**. Within this cohort, average blood glucose levels were 212.5±98.14 mg% and HbA1c was 8.0±0.63%. In addition, serum from healthy individuals was used for reference, and hAAT was used for comparison; all assays were run with 5% FCS and data are presented as change from 5% FCS gap area at each time point. As shown in Figure 8A, conditions that included healthy serum or hAAT depicted an overall improved gap closure. In contrast, serum samples from diabetic patients with poorly-controlled blood glucose appeared to interfere with gap

closure, each individual patient at a unique amplitude (3 representative outcomes are shown). Calculated as AUC **(Fig. 8B)**, as a group, samples from diabetic patients with poorly-controlled blood glucose caused a -20.81±47.18 change in AUC of cell density. Notably, when clinical-grade hAAT was added to sera, cell density increased, and as a group, cell density was elevated at a 77.5±22.64 change in AUC of cell density, a significant change compared to the decline caused by untreated patient serum ($p=0.039$).

Discussion

The effect of a hyperglycemic environment on the properties of hAAT in the context of wound healing was investigated. The premise of the study is that hAAT acts as a binding protein whose properties are compromised by chronic exposure to high glucose levels. This study sheds light on the role of hAAT glycation as part of the pathological mechanism that contributes to the elaboraton of difficult-to-heal wounds in diabetic patients. By this, it is suggested that individuals with poorly controlled glucose levels, at least in the context of tissue injury, may benefit from either topical clinical-grade hAAT or standard hAAT infusion therapy.

That naïve hAAT improves wound repair in the presently tested animal models is consistent with literature (44–50); accordingly, compared to control normoglycemic WT mice, the rate of excisional skin wound closure was enhanced in normoglycemic WT mice treated with topical naïve hAAT and normoglycemic $hAAT^{+/+}$ mice. The mechanism for this attribute of hAAT has yet to be determined, yet there is evidence to support the general concept by which appropriate inflammatory signals prompt the unfolding of inflammatory resolution processes *sooner*, under hAAT-rich conditions (37). Here, on day 3, wound tissue samples exhibited the predominant expression of the anti-inflammatory cytokine IL-1Ra over that of inflammatory IL-1β, while, nonetheless, mature blood vessel formation was by then clearly observed. In contrast, under conditions of hyperglycemia, wounds were generally slow to close in all groups compared to normoglycemic mice. Nonetheless, hyperglycemic $hAAT^{+/+}$ mice and hyperglycemic WT mice treated with topical hAAT exhibited accelerated wound closure. It is speculated that the newly introduced naïve hAAT remains non-glycated in the initial time period after treatment, since the time required for

glycation of any protein is at a scale of days, and, in the case of hAAT, occurs primarily in liver cells (65).

The observation that glycated hAAT fails to accelerate wound repair fits well with its activity as a binding protein that requires intact surface interactions to function (66); however, it is also possible that, like other AGEs, glycated hAAT readily aggravates diabetic wounds. This aspect was investigated by generating glycated hAAT, by exposing hAAT to high levels of glucose over time, under laboratory conditions. Accordingly, its molecular weight increased, it lost its ability to inhibit elastase, and it stimulated macrophage cultures in the absence (and in the presence) of LPS; ultimately, glycated hAAT interfered with epithelial gap closure in-vitro, and with wound closure in-vivo. Mixed with naïve clinical-grade hAAT, the negative impact of gly-hAAT was markedly diminished. In that regard, we asked whether it is possible to rescue the compromised capacity for wound repair contained in serum samples from individuals with poorly controlled blood glucose levels. Thus, in a small scale exploratory clinical arm of the study, clinical-grade hAAT was mixed into serum samples from individuals with poorly controlled blood glucose levels, and then introduced into an epithelial gap closure assay. Without added hAAT, serum from diabetic individuals interfered with the process of re-epithelialization, and serum that was added hAAT displayed enhanced closure rates, suggesting reduction in some of the inherent re-epithelialization limiting factors contained in chronic diabetic serum. Knowingly, this is a qualitative trend and requires a large cohort to fully appreciate and further explore the observed phenomenon.

The wound repair model employed in the present study is not without its limitations. The presence of substantial numbers of myofibroblasts in mice compared to humans contributes to wound contraction (67), for which external tissue holders, such as silicone splints, are sometimes incorporated. Here, to avoid inflicting sutures in hyperglycemic animals, wounds were strategically localized in an anatomical site where natural skin tension is present, thus minimizing spontaneous wound area shrinkage while avoiding the use of external tissue holders.

While AAT improves pancreatic islet β-cell function, animals that received STZ in the present study were confirmed to have reached comparable hyperglycemic values across strains at the time of wounding and throughout follow-up. However, follow-up

duration was limited by hyperglycemia-related mortality. There is the possibility that mouse anti-hAAT antibodies might be induced in WT mice, yet in systemic high-dose treatments the first sign of anti-hAAT antibodies has been shown to occur after 18 days (68); here, the topical application and short duration most probably evade mouse anti-hAAT reaction. The rationale for testing in WT mice alongside the transgenic model is that that a topical approach is more applicable and relevant clinically and also that $hAAT^{+/+}$ mice are exposed to constant, low-levels of ectopic lung-derived circulating hAAT; as such, they do not emulate an infused individual, and as in any genetically engineered animal, might differ from their background WT strain in some nuances that may be derived from a prolonged presence of hAAT. For example, reduced blood pressure has been observed in $hAAT^{+/+}$ mice (69,70). Here, to control for hAAT as a topical protein-based regimen, human serum albumin was used as it is similar in molecular weight and, like hAAT, undergoes passive glycation (71).

In closely examining the processes that underlie the observed improvement in wound closure under hAAT-rich conditions, focus was afforded to inflammation and re-vascularization. According to histology, in the group of hyperglycemic WT mice receiving topical hAAT treatment, mature blood vessels were evident earlier compared to the control group. This finding is consistent with other reports; the group of Bellacen et al. demonstrated acceleration of blood vessel maturation in pancreatic islet allografts (51), and Schuster et al. observed accelerated perfusion of skin flaps in a re-vascularization model (50). In both studies, necrosis was minimized under hAAT-rich conditions. Potential targets of the mechanism of action may include endothelial cells and VEGF. Indeed, hAAT mitigates hypoxia/reoxygenation injury and apoptotic processes in endothelial cells (46,52), and facilitates migration of endothelial cells and mature blood vessel formation, both in the case of diabetic retinopathy (72) and in normoglycemic settings (73). The preservation of a vascular bed has been attributed to hAAT also in the context of AATD, as a blockade of VEGF pathway causes emphysema-like features in mice, even in the presence of intact AAT and without the prototypical infiltration of neutrophils (74). Additionally, VEGF has been linked to some of the beneficial outcomes in systems that test the impact of AAT treatment, as in the case of β islet allograft transplants (75). Interestingly, in a flap re-vascularization model (50), the effect of AAT was only partially impeded by

anti-VEGF co-treatment, suggesting that some benefits of AAT are irrespective of the VEGF pathway. It would be interesting to explore the flap model with hyperglycemia as an underlying condition, with immense implications for surgical limitations in patients with poorly controlled blood glucose levels.

In discussing the potential of VEGF as a molecular responder to hAAT, it is important to distinguish between desired elevated VEGF in normal tissue repair processes, and excessive VEGF in the context of poorly controlled diabetes (76). In the context of the latter, the function of VEGF is altered, a feature that plays a crucial part in the development of diabetic comorbidities (76). Accordingly, in the present study, the hyperglycemic group exhibited significantly elevated VEGF expression levels compared to control mice, aligning with literature regarding serum VEGF levels in patients diagnosed with diabetes compared to normoglycemic individuals (77). In the hAAT group, at the time of sample acquisition on day 3, VEGF expression was lower than in hyperglycemic WT mice, although without reaching statistical significance, but higher than in normoglycemic conditions. Since the trigger for VEGF is primarily hypoxia, this finding might signify improved perfusion of the wounds in the presence of hAAT, as evident by histological analysis. Additionally, AGEs increase VEGF expression and are presumably present at the time of wound analysis.

VEGF expression is also induced by inflammatory conditions, particularly owing to activation of the IL-1 pathway. In that regard, samples from day 3 were examined for IL-1β and IL-1Ra expression, and were found to express significantly greater levels of IL-1Ra in the hAAT group. This outcome sets apart AAT-treated mice from albumin-treated mice, as all hyperglycemic mice displayed elevated IL-1β expression levels. That AAT increases IL-1Ra levels across different conditions is well established (30,32,38), yet the capacity to sustain this desired outcome under hyperglycemic conditions has yet to be reported.

In line with outcomes of changes in the IL-1–pathway under AAT treatment, the expression of genes relating to macrophage polarity was examined. The predominance of M1 macrophages in wound samples under hyperglycemic conditions has been described (10), as was their inhibited transition to M2-like macrophages (6). The failure of hAAT to promote M2-like macrophages in hyperglycemic day-3 wounds stands in contrast to effects of hAAT in normoglycemic

settings (36,78). As expected, hyperglycemic mice exhibited elevated transcript levels of CD14 and MCP-1, irrespective of treatment. This observation may be attributed to the specific time point of sample collection, where the shift towards M2-like cells is at its very beginning even in wounds under normoglycemic conditions (79). It was speculated that mRNA transcripts for CD206 and Arg-1 would precede phenotypic changes on day 3, but it may be that the parameters of the model did not emphasize such early change. Thus, it is possible that the watershed moment for a desired M1-M2 shift occurs at a later time point in hyperglycemic conditions with hAAT treatment. Further experiments are encouraged for assessing macrophage polarization at wound sites under hyperglycemic conditions and hAAT treatment.

A direct glycation of hAAT was performed using standard glycation conditions (80); as a consequence, its size increased in accordance with the expected percent of glycation (59), and its capacity to inhibit elastase activity was clearly compromised. In cell culture systems, glycation of hAAT in the presence of 800 mM glucose was employed, a concentration that provided effective glycation and did not interfere with protein isolation for subsequent testing. Upon introduction to steady-state cell cultures, gly-hAAT induced an inflammatory response, and in LPS-stimulated conditions gly-hAAT further aggravated the inflammatory response, agreeing with the general concept of AGEs (54). Specifically, while native AAT interferes with the TNFα pathway (48,68,81,82) and diminishes IL-6 levels (83), its glycated form induces TNFα levels even in cells that were *not* added LPS. A similar outcome was observed with IL-6 levels (not shown). Importantly, glucose alone did not elicit a pro-inflammatory response, drawing a clear distinction between the effects of glucose and gly-hAAT. The possibility arises that gly-hAAT fails to directly bind to TNFα receptor (TNFR)1 and TNFR2 (66,84). Additionally, lack of protease inhibition by gly-hAAT may contribute to excess protease-dependent release of membrane TNFα by ADAM17 (68).

The question arises as to why doesn’t topical and transgenic hAAT become glycated in the presented experimental systems? hAAT has a relatively short half-life in the bloodstream (3-4 days). Glycation is a process that occurs over time and the extent of glycation increases in association with levels of glucose. Since the synthesis and processing of hAAT occurs primarily in the liver, diabetic individuals experience a higher occurrence of protein glycation whereas the ectopic transgenic

production in hAAT$^{+/+}$ mice has the protein produced in non-hepatocytes (61). In addition, once administered, the turnover of hAAT might be quick enough to evade cumulative glycation.

Gly-hAAT failed to accelerate epithelial gap closure; concomitantly, gly-hAAT caused a delay in wound closure in healthy mice, a delay mitigated to control levels with a rescue intervention using naïve hAAT. Indeed, in ulcers of diabetic patients, hAAT was found to undergo degradation and lose functionality (68), and in the proteome of a diabetic ulcer the levels of hAAT were low compared to an acute wound, thus supporting the possibility that there is relative hAAT *insufficiency* in chronic wounds. Aligning with this concept, serum obtained from individuals with poorly controlled diabetes interfered with epithelial gap closure, an outcome that was diminished by added naïve hAAT. This observation delineates the significant therapeutic potential of hAAT in hyperglycemic environments.

Based on the present study, future studies are encouraged; for example, it is imperative to explore pig skin models due to their closer resemblance to human skin. In addition, long-term studies with hyperglycemic mice, potentially using less severe models of hyperglycemia or genetically modified mice that allow for extended follow-up periods without the high mortality rates, should be performed. This would provide a more comprehensive collection of data on wound healing processes. There is also a need to further investigate the properties and effects of glycated hAAT, such as protein interactions. At the cellular target level, further experiments may focus on macrophage polarization in hyperglycemic wounds treated with gly-hAAT.

In conclusion, the modulatory capacities of hAAT underscore its ability to navigate between pro-inflammatory and pro-resolution states based on local molecular signals. Our research emphasizes that this modulation is crucial, especially in the nuanced landscape of hyperglycemic conditions where inflammatory states are prevalent. The elucidation that exogenous administration of naïve hAAT harbors therapeutic benefits holds the potential to not only counteract the pro-inflammatory attributes incited by glycation, but also to re-establish a more physiological environment for optimal wound healing.

Author Contributions:

Conceptualization: I.F, A.N, Y.I, E.Z and E.C.L.; methodology: I.F., A.N, Y.I, M.Z., A.T, L.S, R.S. and D.H; writing—original draft preparation: I.F; writing—review and editing: E.C.L. and E.S.; visualization: I.F. and E.C.L.; supervision: E.C.L. and E.S.; funding acquisition: E.C.L. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The data presented in the present study are available upon reasonable request from the corresponding author.

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