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Biomarkers

Glucagon-Like Peptide-1 Is a Marker of Systemic Inflammation in Patients Treated with High-Dose Chemotherapy and Autologous Stem Cell Transplantation

Maria Schou Ebbesen^{1,*}, Hannelouise Kissow^{2,3}, Bolette Hartmann^{2,3}, Kathrine Grell^{1,4}, Jette Sønderkov Gørløv⁵, Katrine Kielsen^{1,6}, Jens Juul Holst^{2,3}, Klaus Müller^{1,6}

¹ Department of Pediatrics and Adolescent Medicine, University Hospital Rigshospitalet, Copenhagen, Denmark

² Department of Biomedical Sciences, University of Copenhagen, Copenhagen, Denmark

³ NNF Center for Basic Metabolic Research, University of Copenhagen, Copenhagen, Denmark

⁴ Section of Biostatistics, Department of Public Health, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark

⁵ Department of Hematology, University Hospital Rigshospitalet, Copenhagen, Denmark

⁶ Institute for Inflammation Research, University Hospital Rigshospitalet, Copenhagen, Denmark

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Autologous stem cell transplantation (ASCT) is challenged by side effects that may be propagated by chemotherapy-induced mucositis, resulting in bacterial translocation and systemic inflammation. Because gastrointestinal damage appears as an early event in this cascade of reactions, we hypothesized that markers reflecting damage to the intestinal barrier could serve as early predictive markers of toxicity. Glucagon-like peptide-1 (GLP-1), a well-known regulator of blood glucose, has been found to promote intestinal growth and repair in animal studies. We investigated fasting GLP-1 plasma levels in 66 adults undergoing ASCT for lymphoma and multiple myeloma. GLP-1 increased significantly after chemotherapy, reaching peak levels at day +7 post-transplant (median, 8 pmol/L [interquartile range, 4 to 12] before conditioning versus 10 pmol/L [interquartile range, 6 to 17] at day +7; $P = .007$). The magnitude of the GLP-1 increase was related to the intensity of conditioning. GLP-1 at the day of transplantation (day 0) was positively associated with peak C-reactive protein (CRP) levels (46 mg/L per GLP-1 doubling, $P < .001$) and increase in days with fever (32% per GLP-1 doubling, $P = .0058$). Patients with GLP-1 above the median at day 0 had higher CRP levels from days +3 to +10 post-transplant than patients with lower GLP-1 ($P \leq .041$) with peak values of 238 versus 129 mg/L, respectively. This study, which represents the first clinical investigation of fasting GLP-1 in relation to high-dose chemotherapy, provides evidence that GLP-1 plays a role in regulation of mucosal defenses. Fasting GLP-1 levels may serve as an early predictor of systemic inflammation and fever in patients receiving high-dose chemotherapy.

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INTRODUCTION

High-dose chemotherapy with autologous stem cell transplantation (ASCT) is a potentially curative treatment of many hematologic malignancies [1] but is challenged by severe side effects and complications such as mucositis, organ toxicity, and infections [2,3]. In most patients undergoing ASCT, varying degrees of systemic inflammation are seen during the first couple of weeks after conditioning with chemotherapy. This inflammatory response is initiated because of chemotherapy-induced injury to the mucosal barrier of the digestive tract

[4–8], which increases intestinal permeability and translocation of bacterial products into the bloodstream [5,7,9,10]. Many previous studies have suggested that this inflammatory response plays a key role in induction of treatment-related complications and results in poor prognosis [11–16].

C-reactive protein (CRP) is a reliable marker for systemic inflammation after chemotherapy [17] and has been found to reach maximum levels about 7 to 14 days after infusion of stem cells [9,12,18,19]. This peak coincides with maximum intensity of mucositis as measured by clinical scoring, increased intestinal permeability, and a decline in citrulline, a marker of functioning enterocytes [4,12,20]. Importantly, the magnitude of the CRP response is associated with severe complications such as mucositis, pneumonia, bacteremia, *Clostridium difficile* colitis, and other infections, with potentially fatal outcome in patients undergoing ASCT [12,21]. However, the

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* Correspondence and reprint requests: Maria S. Ebbesen, Department of Pediatrics and Adolescent Medicine, University Hospital Rigshospitalet, Blegdamsvej 9, 2100 Copenhagen, Denmark.

E-mail address: maria.ebbe@gmail.com (M.S. Ebbesen).

degree of inflammation is quite variable between patients, even among patients receiving the same conditioning regimen. It is not well understood why some patients are more prone to respond with severe inflammation than others, and we have at present no biomarkers that with sufficient precision can predict the inflammatory response in individual patients, limiting the possibility of personalized prophylaxis with antibiotics and other early interventions.

Continued proliferation and regeneration of intestinal epithelium is essential for maintenance of the mucosal barrier and involves peptide hormones produced in the gut [22,23]. Glucagon-like peptide-1 (GLP-1) and GLP-2 are hormones co-secreted from intestinal enteroendocrine L cells in response to food intake and play a major role in regulating blood glucose through stimulation of insulin secretion (GLP-1) and promotion of nutrient absorption via expansion of the mucosal epithelium (GLP-2) [24–26]. However, increased secretion of both hormones is also seen after intestinal injury induced by chemotherapy in mice and rats [27–30] as well as in gut ischemia and inflammatory bowel disease in humans [31,32]. Importantly, GLP-2 and/or GLP-1 were found to have a trophic effect on the small intestine in several studies in rodents [33–37]. Additionally, GLP-2 has been shown to stabilize the integrity of intestinal epithelial barrier [38–41], and both GLP-1 and GLP-2 administration reduces chemotherapy-induced intestinal injury [27,42–45].

Based on these studies we hypothesized that elevated levels of GLP-1 could be an early marker of gut injury and potentially predict treatment-related complications in patients undergoing high-dose chemotherapy. The present study represents the first investigation of fasting GLP-1 plasma levels at consecutive time points in patients treated with high-dose chemotherapy followed by ASCT. Our findings indicate that increased levels of GLP-1 may serve as an early predictor of systemic inflammation and infection in these patients.

METHODS

Study Population

We prospectively included 66 patients (ages 20 to 72 years) undergoing ASCT at University Hospital Rigshospitalet, Copenhagen, Denmark from February 2015 to October 2016. Written and oral informed consent was obtained for all included patients. The study was approved by the local ethics committee (H-7-2014-016) and conducted in accordance with the Declaration of Helsinki. Inclusion criteria were patients scheduled for ASCT, age older than 18 years, and patient informed consent. Of 68 eligible patients, 2 were lost to follow-up before 3 weeks after transplantation due to transfer to other hospitals and therefore were excluded from the study.

The choice of pretransplant conditioning was based on the diagnosis in accordance with international guidelines, with all patients with myelomas receiving conditioning regimen 1 and all patients except 2 with lymphoma receiving conditioning regimen 2 (Table 1). All patients received oral antimicrobial prophylaxis consisting of ciprofloxacin, coamoxiclav, and fluconazole from day +4. Intravenous treatment with piperacillin/tazobactam or meropenem antibiotics was given based on daily measurements of body temperature and was administered according to a standardized regimen during days with fever (>38.5°C) until body temperature declined below 38.5°C. Body temperature was measured daily.

Inflammatory Parameters

CRP was monitored routinely in all patients every other day as a minimum during the first 3 weeks post-transplantation, and extra measurements were taken at the discretion of the physician in charge. When more than 1 measurement per day per patient was available, we calculated the mean to represent the CRP level of that day. Post-transplantation CRP_{max} was defined as the maximum CRP value from day +1 to day +21. CRP was analyzed using Modular P Module (Roche, Basel, Switzerland) (upper normal limit, 10 mg/L) at the Department of Clinical Biochemistry, University Hospital Rigshospitalet. Blood cultures were routinely collected on all patients with fever before initiation of i.v. antibiotic treatment, and results were registered from day –14 to day +30.

Table 1
Patient Characteristics and Treatment Modalities (N = 66)

Characteristics	Value
Median age, yr (range)	56 (20–72)
Sex	
Male	41 (62)
Female	25 (38)
Diagnosis	
Multiple myeloma	33 (50)
Non-Hodgkin lymphoma	29 (44)
Hodgkin lymphoma	3 (5)
Plasma cell leukemia	1 (2)
Conditioning regimen	
Melphalan high dose +/- carfilzomib/bortezomib	33 (50)
BEAM	30 (45)
Ara-C + CP + VP16 + thiotepa + bortezomib + lenalidomide	1 (2)
BCNU + thiotepa + rituximab	2 (3)

Values are n (%) unless otherwise defined. Ara-C indicates cytarabine; BCNU, carmustine; VP16, etoposide; CP, cyclophosphamide; BEAM, Ara-C + BCNU + VP16 + melphalan.

Quantification of GLP-1

Blood samples were collected at 5 time points during ASCT: before initiation of the conditioning regimen (baseline), at the day of transplantation before stem cell infusion (day 0), and at days +7, +14, and +21 post-transplantation. EDTA anticoagulated blood was centrifuged within 2 hours after collection, and plasma was isolated and stored at –80°C until analysis.

The plasma concentration of GLP-1 was measured in duplicates using a total GLP-1 ELISA (cat. no. 10-1278-01; Mercodia, Uppsala, Sweden) according to the manufacturer's instructions. This ELISA measures both active GLP-1 (7–36) amide and the degraded isoform GLP-1 (9–36) amide and reflects the secretion of GLP-1 because amidated isoforms of GLP-1 are highly predominant in humans [46]. Measurement range was .9 to 940 pmol/L.

Statistical Analyses

The associations of GLP-1 over time and with patient-specific characteristics (conditioning regimen, age, sex) were analyzed with a mixed model with a compound symmetry covariance matrix, and the significance was assessed with a Wald test with the degrees of freedom calculated using the Satterthwaite approximation. GLP-1 was log-transformed due to its skewness. Similarly, when investigating the association between CRP over time and dichotomized GLP-1 level at day 0. The Mann-Whitney U-test was used for comparison of continuous variables between groups. Simple and adjusted linear regression models were used to determine the association between GLP-1 day 0 (log 2-transformed) and CRP and negative binomial regression models to determine the association with days with fever. In subanalyses where conditioning was included as a covariate, only the 63 patients in either group 1 (high-dose melphalan, with or without bortezomib or carfilzomib) or group 2 (cytarabine in combination with carmustine, etoposide, and melphalan) were included.

A 2-sided $P < .05$ was considered statistically significant. All statistical analyses were performed using R statistical software version 2.15.3 (R Foundation for Statistical Computing, Vienna, Austria) and RStudio (RStudio, Boston, MA).

RESULTS

Clinical characteristics of the included patients are listed in Table 1.

GLP-1 Levels

The median GLP-1 level before conditioning for all included patients was 7.7 pmol/L (interquartile range, 4.3 to 11.5), which is within the range of the 95% confidence interval (CI) of fasting levels of 774 healthy individuals (1 to 21 pmol/L) [47]. Overall, the association between GLP-1 levels and measurement time points was significant ($P = .010$), and GLP-1 levels increased significantly from before conditioning to day 0 (33%; 95% CI, 3 to 70; $P = .027$) and peaked at day +7 (41%; 95% CI, 10 to 81; $P = .0073$) (Figure 1A).

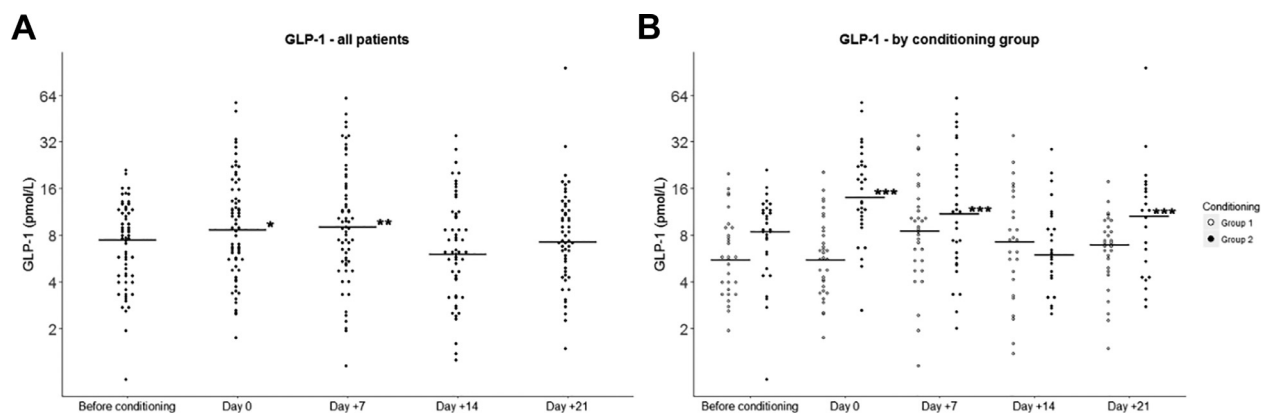


Figure 1. GLP-1 levels during ASCT from before start of conditioning until 21 days post-transplant. Day 0 refers to the day of transplantation after conditioning with high-dose chemotherapy. Horizontal line indicates median GLP-1. (A) All included patients. Statistical evaluation indicates GLP-1 levels compared with preconditioning levels (* $P = .03$; ** $P = .007$). (B) Patients stratified by conditioning group. Patients conditioned with cytarabine in combination with carmustine, etoposide, and melphalan (group 2) had generally higher GLP-1 levels than patients treated with high-dose melphalan with or without bortezomib or carfilzomib (group 1). Statistical evaluation indicates differences between the 2 conditioning groups (*** $P < .001$ [day 0]; $P = .048$ [day 7]; $P = .03$ [day +21]).

GLP-1 level before conditioning was not associated with sex ($P = .38$), age ($P = .11$), or diagnosis ($P = .061$). GLP-1 was significantly associated with conditioning group ($P < .0001$) with levels at days 0, +7, and +21 significantly higher in conditioning group 2 (patients with lymphoma) than in group 1 (patients with multiple myeloma) (155% [95% CI, 78 to 465; $P < .0001$], 44% [95% CI, .3 to 108; $P = .048$], and 54% [95% CI, 2 to 131; $P = .03$], respectively) (Figure 1B). Moreover, overall GLP-1 was significantly lower for older patients (11% per 10 years; 95% CI, 3 to 18; $P = .013$) but was not associated with sex ($P = .35$).

Inflammatory Response

Of the 48 patients with CRP measured at day 0, 44 (92%) had CRP day 0 within normal range (≤ 10 mg/L). Post-transplantation median CRP increased and reached a peak at day +9 after transplantation (Figure 2A), with daily median levels from day +2 and onward significantly higher than CRP day 0 (all $P < .0017$). Median post-transplant CRP_{max} was 181 mg/L (interquartile range, 61 to 266).

Fifty-two patients (79%) experienced fever $> 38.5^\circ\text{C}$ during the first 3 weeks post-transplant. The median duration of fever was 8 days (interquartile range, 3 to 12). At day +11 the highest number of patients experiencing fever was seen (47 [71%]) (Figure 2B).

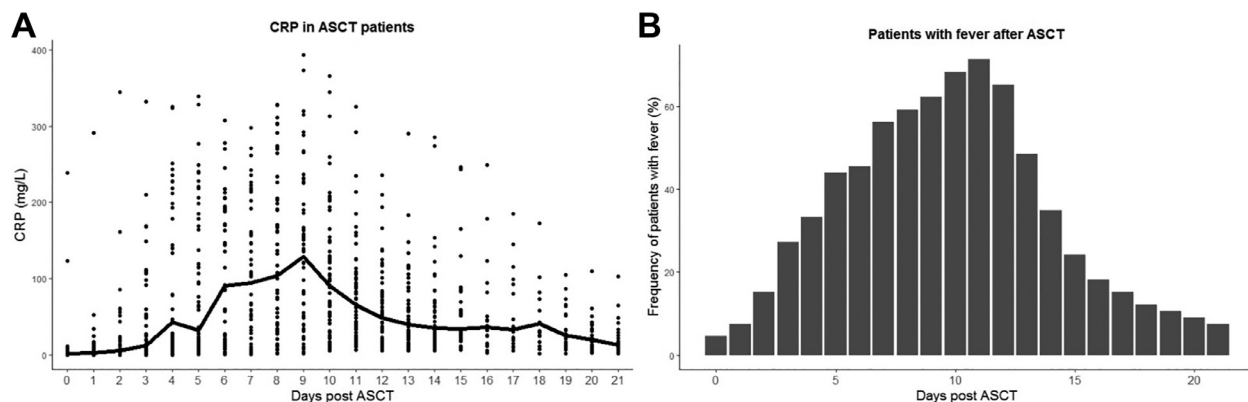


Figure 2. CRP and fever during treatment. CRP levels (A) and frequency of patients with fever (B) after high-dose chemotherapy and stem cell infusion. Black line in A indicates median CRP level.

GLP-1 and Inflammation

We investigated associations between GLP-1 and CRP and fever. A doubling in GLP-1 at day 0 was associated with an increase of 46 mg/L in CRP_{max} (95% CI, 24 to 68; $P = .00018$) (Figure 3A) and an increase of 32% in days with fever (95% CI, 8 to 62; $P = .0058$) (Figure 3B). When adjusting for conditioning group the associations with GLP-1 at day 0 remained significant ($P = .024$ and $P = .042$, respectively). In simple regression analyses, conditioning group 2 had on average 80 mg/L higher CRP_{max} compared with group 1 (95% CI, 22 to 138; $P = .0091$) and almost twice as many days with fever (96%; 95% CI, 27 to 201; $P = .022$). By comparing CRP in 2 groups stratified by the median values of GLP-1 at day 0 (9 pmol/L), we could demonstrate higher levels of CRP from days +3 to +10 in those with high GLP-1 values on day 0 (all $P \leq .041$) (Figure 4).

Infections

Only 2 patients presented with a positive blood culture (both post-transplant), which did not allow investigation of association between documented infections and GLP-1 levels.

Survival

Nine patients (14%) died during 1 year of follow-up, and 2 of these died because of treatment-related complications. These limited numbers did not allow conclusions regarding prediction of survival by GLP-1 measurements.

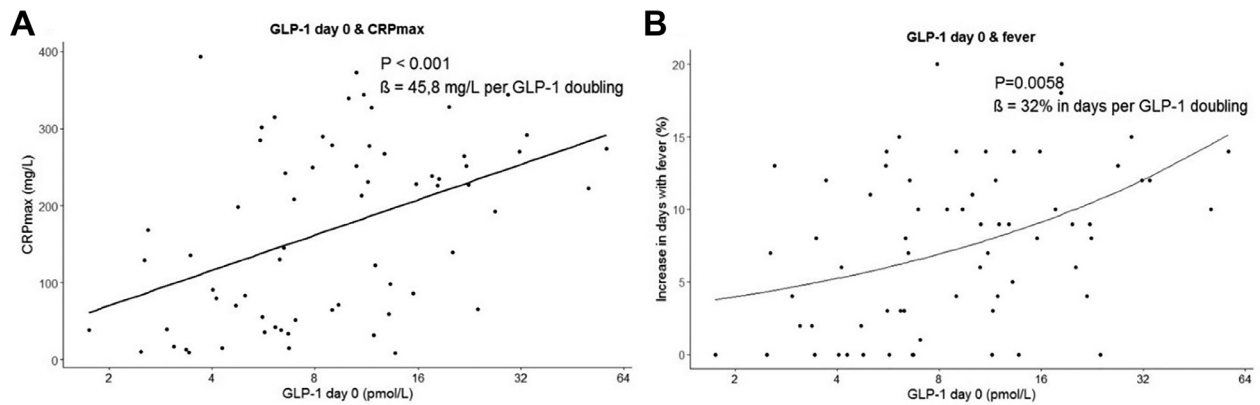


Figure 3. GLP-1 day 0 associations with inflammation after transplantation. Associations between GLP-1 levels at day 0 and inflammatory response: CRP_{max} (A) and days with fever (B) after ASCT. *P* values by simple linear regression and negative binomial regression analyses showed a significant association between GLP-1 levels at day 0 and inflammatory response.

DISCUSSION

In this study we investigated fasting GLP-1 levels in the early toxic phase in patients undergoing ASCT after high-dose chemotherapy. Our data indicate that fasting levels of GLP-1 are increased after chemotherapy, peaking around day +7 after the transplant, and, importantly, our data indicate that high levels of GLP-1 shortly after the completion of high-dose chemotherapy are predictive of subsequent increases in CRP during the first 3 weeks after transplantation.

GLP-2 is a well-known growth factor for intestinal epithelium, stimulating proliferation of crypt cells while inhibiting apoptosis of enterocytes in the small intestine [26,33,34,48–50]. A similar trophic effect of GLP-1 has been suggested by studies on rats [35,51] and mice [36,52] showing small intestinal and colonic growth after treatment with GLP-1 analogues. Furthermore, endogenous GLP-1 contributes to intestinal

epithelial recovery after chemotherapy in mice [27], whereas loss of the GLP-1 receptor increases the severity of intestinal injury [53].

GLP-1 may also act as an anti-inflammatory peptide attenuating both local and systemic inflammation [54,55] by improving mucosal integrity through interactions with intestinal intraepithelial lymphocytes, found to express functional GLP-1 receptors [53,56]. In addition, GLP-1 has stimulatory effects on innate immune mechanisms, including production of alpha-defensin by Paneth cells [57]. GLP-1 treatment has shown anti-inflammatory effects by suppressing proinflammatory cytokines such as tumor necrosis factor- α , IL-6, and IL-1 β in intestinal mucosa in mice with colitis [58–60] and in humans with diabetes type 1 and 2 and psoriasis [61–63]. Based on these findings, it may be hypothesized that increased level of GLP-1 in response to chemotherapy represents a protective

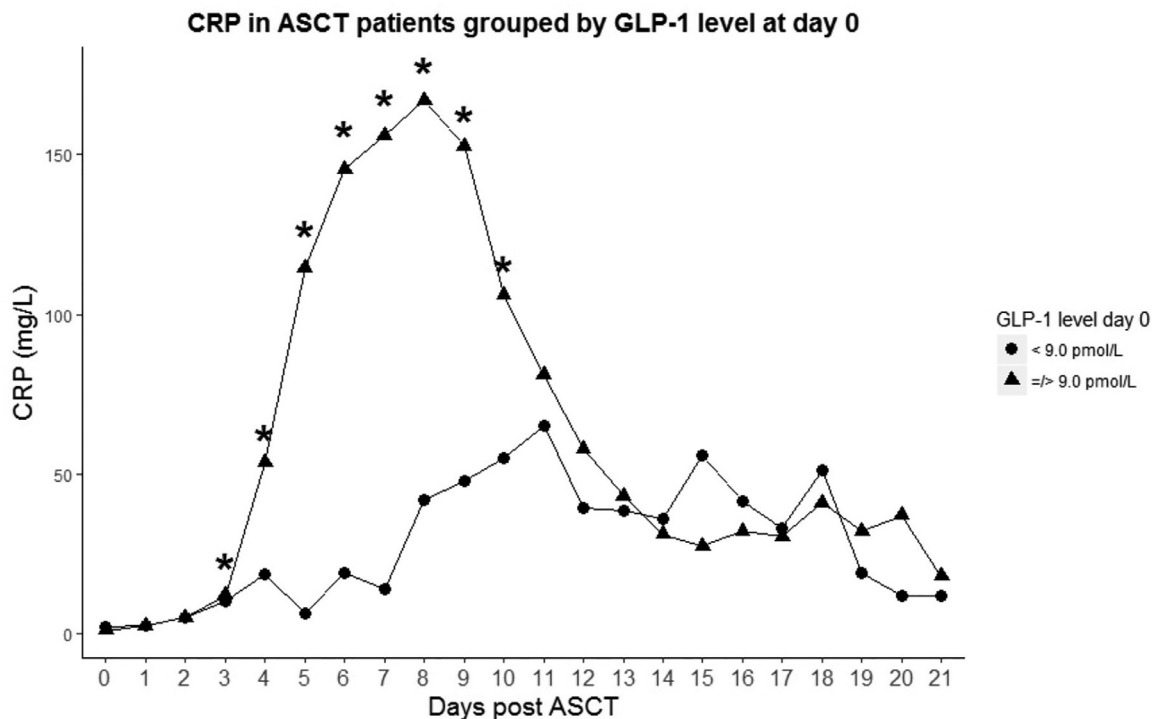


Figure 4. CRP after transplantation by GLP-1 increase at day 0. Median CRP levels after ASCT in 2 groups stratified according to the median levels of GLP-1 at day 0. Asterisk indicates statistical difference in CRP levels between the 2 groups (all $P \leq .041$).

reactive mechanism that may help to limit tissue damage and fasten recovery of epithelial integrity, thereby limiting inflammation.

To our knowledge we are the first to explore fasting GLP-1 levels in human subjects after chemotherapy treatment. However, our findings are in line with findings by Kissow et al. and others showing increased levels of both GLP-1 and GLP-2 after chemotherapy in rats [27] and mice [28–30]. Other studies found increased GLP-1 or GLP-2 levels after nonchemotherapy-related gut injury in humans [31,32] and animals [31], lending support to the notion that these hormones are secreted in response to intestinal injury.

Further evidence of such a protective effect is found in studies by Hytting et al. [30] showing that ablation of L cells in chemotherapy-treated mice leads to severe mucositis and insufficient intestinal healing, whereas chemotherapy-treated mice with normal function of L cells show compensatory hyperproliferation of intestinal epithelial cells in association with a marked increase in GLP-2 secretion. This is in line with data from Kissow et al. [27] showing similar findings when antagonizing the GLP-1 receptor selectively. Furthermore, Hytting et al. showed that co-treatment with both GLP-1 and GLP-2 analogues was more effective than a single GLP-1 analogue for rebuilding intestinal epithelium and maintaining body weight in mice.

The mechanism behind the increase in GLP-1 after gut injury is not fully understood, but data from Lebrun et al. [31] suggest that innate responses to bacterial lipopolysaccharide (LPS) may play a role in the induction of GLP-1 secretion. Accordingly, enteroendocrine L cells are stimulated by LPS through interaction with Toll-like receptor 4 after gut injury, leading to a rapidly increased secretion of GLP-1 [31]. Other studies in experimental animals confirmed increased GLP-1 secretion in response to LPS [64,65], apparently mediated through proinflammatory cytokines including IL-6 [64,66] and tumor necrosis factor- α [31,64,67]. Likewise, in humans Lebrun et al. [31] found GLP-1 secretion after LPS administration in volunteers. These authors also reported increased GLP-1 levels after induction of ischemia in the human intestine in vivo, suggesting a close relationship between gut inflammation and GLP-1 secretion in humans.

Breaks in the mucosa after chemotherapy have been found to serve as portals of entry for microorganisms, leading not only to bacteremia but also to penetration of pathogen-associated molecular patterns and endogenous danger-associated molecular patterns, produced as a result of cell death [68–70]. The resulting inflammatory response has been found to be predictive of organ toxicity and transplant-related mortality in allogeneic hematopoietic SCT [5,14–16,20,71,72], and several studies confirm a relationship between chemotherapy-induced mucosal barrier injury and systemic inflammation [4,8,9,19,73]. Our finding of a positive association between CRP and GLP-1 is in line with these results. Altogether, these studies suggest that increased levels of GLP-1 during chemotherapy may be induced by LPS in combination with an initial release of inflammatory cytokines.

Individuals with type 2 diabetes mellitus have a diminished meal-related GLP-1 secretion [74], and we could speculate this lower protection from GLP-1 after meals makes the intestinal epithelium more vulnerable after chemotherapy, resulting in higher risk of mucositis and severe systemic inflammation. Furthermore, it is not known if a diminished meal-related GLP-1 secretion makes the L cells less responsive to other secretory stimuli as well, potentially making the diabetes diagnosis a confounder for our results. Eight patients in our cohort were

diagnosed with type 2 diabetes at the time of transplant, and these patients did not differ from the rest of the study population in GLP-1 levels or degree of systemic inflammation.

Nutrient ingestion is the primary physiologic stimulatory signal for GLP-1 and GLP-2 secretion [25,75]. Previous studies in rats [48] and mice [76] showed that fasting for 1 or a couple of days resulted in atrophy of the small intestine but could be reversed by refeeding and that this refeeding adaptation was prevented upon antagonizing the GLP-2 receptor. Other studies found that intestinal atrophy seen in rodents and pigs after total parenteral nutrition [48,77] could be ameliorated by co-infusion of GLP-2 [41,77,78]. Because patients undergoing high-dose chemotherapy treatment often have low or no enteral nutrition for days during the first few weeks after transplantation, the direct toxic effects of chemotherapy could be aggravated through insufficient GLP-1 and GLP-2 secretion, leading to incomplete healing and regeneration of the intestinal epithelium. Accordingly, further studies should focus on the role of enteral feeding in maintaining gut integrity through induction of GLP-1 and GLP-2. The variation in GLP-1 levels seen both pre- and post-transplant, even after stratification into conditioning regimens, demonstrates that additional factors may influence fasting plasma GLP-1 levels in humans, indicating a need for further studies to fully understand the potential of GLP-1 as a prognostic marker in clinical settings.

In conclusion, our data indicate that high-dose chemotherapy leads to increased fasting levels of GLP-1 and that these are predictive of increased systemic inflammation, adding to the mounting evidence that GLP-1 plays an important role in regulation of the mucosal defense. GLP-1 should be further investigated as a potential early predictive biomarker of treatment-related morbidity.

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