The Sodium Glucose Cotransporter Type 2 Inhibitor Empagliflozin Preserves β-Cell Mass and Restores Glucose Homeostasis in the Male Zucker Diabetic Fatty Rat

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ABSTRACT

Type 2 diabetes is characterized by impaired β-cell function associated with progressive reduction of insulin secretion and β-cell mass. Evidently, there is an unmet need for treatments with greater sustainability in β-cell protection and antidiabetic efficacy. Through an insulin and β cell–independent mechanism, empagliflozin, a specific sodium glucose cotransporter type 2 (SGLT-2) inhibitor, may potentially provide longer efficacy. This study compared the antidiabetic durability of empagliflozin treatment (10 mg/kg p.o.) against glibenclamide (3 mg/kg p.o.) and liraglutide (0.2 mg/kg s.c.) on deficient glucose homeostasis and β-cell function in Zucker diabetic fatty (ZDF) rats. Empagliflozin and liraglutide led to marked improvements in fed glucose and hemoglobin A1c levels, as well as impeding a progressive decline in insulin levels. In contrast, glibenclamide was ineffective. Whereas the effects of liraglutide were less pronounced at week 8 of treatment compared with week 4, those of empagliflozin remained stable throughout the study period. Similarly, empagliflozin improved glucose tolerance and preserved insulin secretion after both 4 and 8 weeks of treatment. These effects were reflected by less reduction in β-cell mass with empagliflozin or liraglutide at week 4, whereas only empagliflozin showed β-cell sparing effects also at week 8. Although this study cannot be used to dissociate the absolute antidiabetic efficacy among the different mechanisms of drug action, the study demonstrates that empagliflozin exerts a more sustained improvement of glucose homeostasis and β-cell protection in the ZDF rat. In comparison with other type 2 diabetic treatments, SGLT-2 inhibitors may through insulin-independent pathways thus enhance durability of β-cell protection and antidiabetic efficacy.

Introduction

The prevalence and incidence of type 2 diabetes mellitus (T2DM) are increasing worldwide with the concurrent epidemic of obesity undoubtedly providing the strongest drive to trigger hyperglycemia (Pi-Sunyer, 2009). T2DM is characterized by high blood glucose caused by insulin resistance, followed by insulin deficiency due to impaired pancreatic β-cell function and survival (Kahn et al., 2006). Currently approved agents used to control hyperglycemia in T2DM are specifically addressing the underlying endocrine pathogenesis by increasing insulin secretion or insulin sensitivity, thus relying on residual β-cell function as exemplified by sulfonylureas (e.g., glibenclamide), glucagon-like peptide-1 (GLP-1) analogs (e.g., liraglutide), and metformin. In addition to being dependent on insulin action, many blood glucose–lowering agents cause hypoglycemia, weight gain, or gastrointestinal discomfort. Consequently, the limitations and side effects of these agents have spurred the research in optimizing metabolic control by specifically targeting renal glucose reabsorption through pharmacological inhibition of the sodium glucose cotransporter type-2 (SGLT-2). SGLT-2 is almost entirely confined to the early segment of the renal proximal tubule, where it mediates reabsorption of most of the filtered glucose (Ghosh et al., 2012). Many T2DM patients show increased glucose reabsorption, which may be associated with upregulated SGLT-2 function (Rahmoune et al., 2005), thus further suggesting the feasibility in targeting SGLT-2 function to control hyperglycemia. Furthermore, as the mechanism of action of SGLT-2 inhibitors is independent of β-cell dysfunction or severity of insulin resistance, their efficacy is not expected to decline in the presence of severe insulin resistance or progressive β-cell failure. Consequently, several SGLT-2 inhibitors are in clinical development for management of hyperglycemia in T2DM (Bailey, 2011).

Empagliflozin is a potent competitive SGLT2 inhibitor with high selectivity to SGLT-2 over other SGLT isoforms (Luippold et al., 2012). Empagliflozin effectively increases urinary glucose excretion, reduces blood glucose and hemoglobin A1c (HbA1c) levels, improves glucose tolerance, and increases insulin sensitivity in male Zucker diabetic fatty (ZDF) rats and db/db mice without producing hypoglycemia (Kern et al., 2012; Thomas et al., 2012). In accordance, recent clinical trials have confirmed its tolerability and efficacy in reducing fasting and postprandial glucose and HbA1c levels in T2DM patients

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ABBREVIATIONS: AUC, area under the curve; GLP-1, glucagon-like peptide-1; HbA1c, hemoglobin A1c; OGTT, oral glucose tolerance test; SGLT-2, sodium glucose cotransporter type 2; T2DM, type 2 diabetes mellitus; ZDF, Zucker diabetic fatty.

(Rosenstock et al., 2013; Sarashina et al., 2013). To the extent that prolonged hyperglycemia contributes to glucotoxicity-induced deterioration of β-cell function and accelerated β-cell loss in T2DM (Stolar, 2010), it may be hypothesized that the antihyperglycemic effect of empagliflozin is associated with prevention, or alternatively delayed onset, of β-cell mass depletion. Hence, this study aimed to characterize the durability of empagliflozin on glucose homeostasis, as compared with liraglutide and glibenclamide, and whether empagliflozin treatment would lead to preservation of β-cell mass over time in male ZDF rats.

Materials and Methods

Animals. A total of 100 male ZDF rats was obtained from Charles River Labs (Sulzfeld, Germany). Animals were scheduled to arrive at the Gubra stables at 6–7 weeks of age. Prior to arrival, the rats were on chow (Purina 5008; St. Louis, MO) from weaning age. Rats were housed two per cage under a 12-hour light/dark cycle at controlled temperature conditions with ad libitum access to chow (Purina 5008) and water. All animal experiments were conducted in accordance with internationally accepted principles for the care and use of laboratory animals and were approved by the Danish Committee for animal research (license 2008/561-1565).

Compound Treatment. According to fed blood glucose (17.3 ± 0.6 mM) on the day before study start, 80 animals (9–10 weeks of age) being closest to the overall mean were randomized into four groups of

$n = 20$ per group. Eight additional animals were terminated, constituting the baseline group. Animals were dosed once daily before lights out with vehicle (1% hydroxypropyl methylcellulose; Sigma-Aldrich, St. Louis, MO), empagliflozin (10 mg/kg, 5 ml/kg p.o.), liraglutide (200 μg/kg, 2 ml/kg s.c.), or glibenclamide (3 mg/kg, 5 ml/kg p.o.). Liraglutide (dissolved in phosphate-buffered saline) was gradually titrated over the first 4 days until reaching the 200 μg/kg per day dose. At experimental day 26, each group was re-randomized according to fed blood glucose levels into two subgroups of $n = 10$. One subgroup was subjected to the oral glucose tolerance test (OGTT) day 28 and terminated 3 days after. The other subgroup continued dosing for an additional 4 weeks, followed by an OGTT day 56.

Blood Biochemistry (Glucose, Insulin, HbA1c) and Oral Glucose Tolerance Test. Blood samples for determination of weekly blood glucose and insulin concentration were taken from the sublingual plexus of conscious, nonfasted animals in the morning. Glucose samples were collected into 10 μl heparinized glass capillary and analyzed on the test day using a BIOSEN c-Line glucose analyzer. Insulin samples (75 μl blood) were collected into heparinized tubes, and plasma-separated samples were analyzed using an AlphaLisa (PerkinElmer, Skovlunde, Denmark) with a commercial enzyme-linked immunosorbent assay kit (Mercodia, Uppsala, Sweden), according to instructions. HbA1c was measured at weeks 2, 4, 6, and 8 using a Cobas c-111 autoanalyzer with a commercial kit (Roche Diagnostics, Mannheim, Germany), according to instructions. The OGTTs were carried out in the morning at experimental days 28 and 56 following a mild overnight fast (50% of their average 24-hour intake). Animals were dosed with compound 45 minutes prior to receiving the oral glucose load (2.0 g/kg,
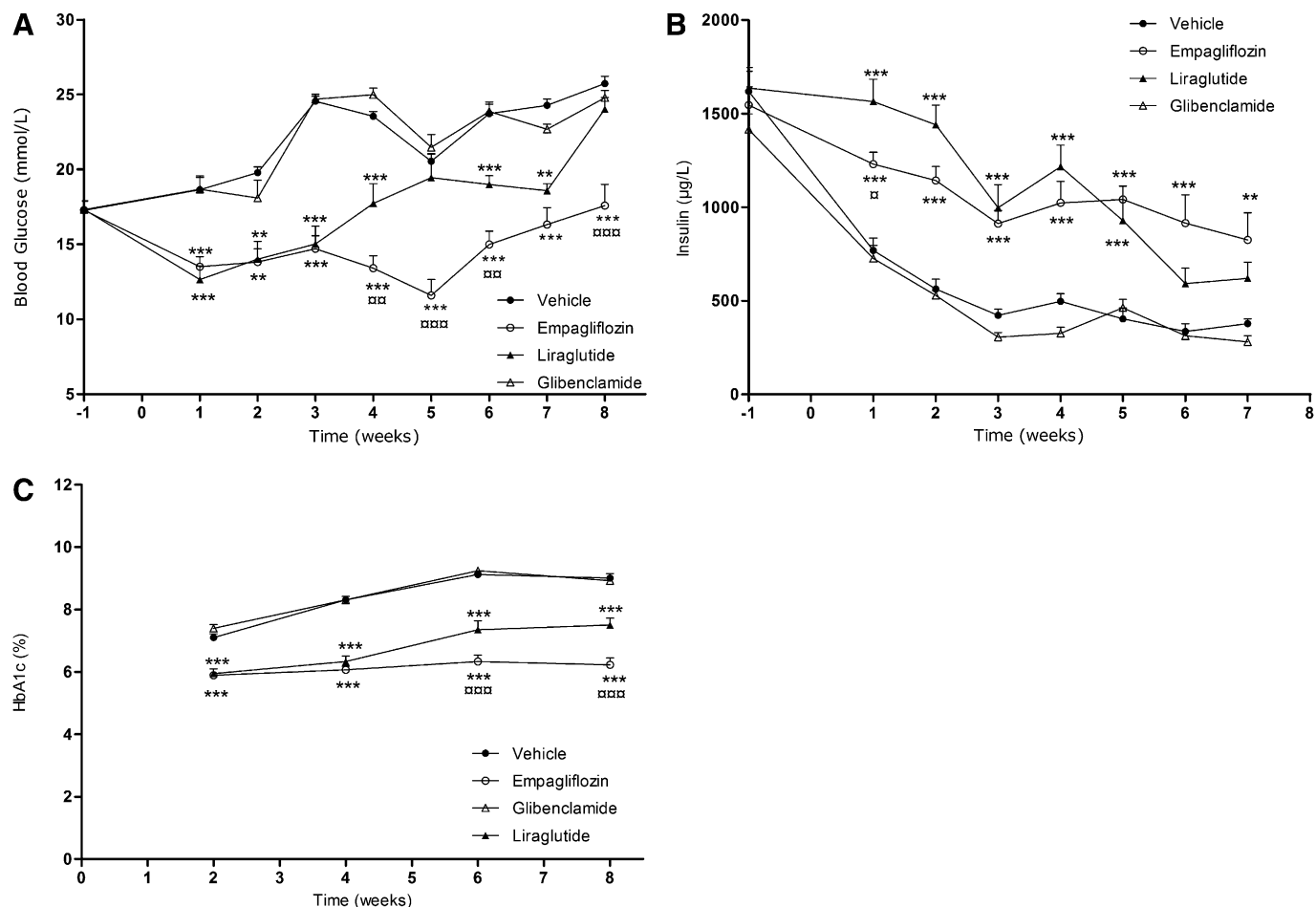
Fig. 1. Effects of empagliflozin, liraglutide, and glibenclamide on blood glucose (A), plasma insulin (B), and HbA1c (C) levels in male ZDF rats. **$P < 0.01$; ***$P < 0.001$ (compared with vehicle control group); $^{\circ}P < 0.05$; $^{\circ\circ}P < 0.01$; $^{\circ\circ\circ}P < 0.001$ (compared with liraglutide).

4 ml/kg). Blood samples were taken from the sublingual capillaries at time points −60, 0, 15, 30, 60, 120, and 240 minutes after the oral glucose administration. Plasma for insulin was sampled at the same time points. Rats were terminated 3 days following the OGTTs.

Islet Immunohistochemistry. The pancreata were removed en bloc at termination, immersion fixed in 4% formaldehyde (4% formaldehyde in 0.1 M phosphate buffer; phosphate-buffered saline, pH 7.4), and stored at 4°C until further processing. Determination of β-cell mass was performed, as described previously (Paulsen et al., 2010). Briefly, the pancreas was rolled into a cylinder, infiltrated in paraffin overnight, and cut into eight 8–10 transverse slabs. The slabs were embedded on their cut surface in two blocks of paraffin, and one 5-μm top section from each block was arranged on one object glass representing in total a systematic uniform random sample of the complete pancreas. Immunohistochemistry was performed using a primary non–β-rabbit antibody cocktail consisting of anti-glucagon (1:1000; Phoenix Pharmaceuticals, Burlingame, CA), anti-somatostatin (1:1600; DakoCytomation, Glostrup, Denmark), and anti-pancreatic polypeptide (1:1000; Euro Diagnostica, Malmoe, Sweden), followed by a secondary biotinylated donkey anti-rabbit antibody (1:2000; Jackson ImmunoResearch Laboratories, West Grove, PA) and horseradish peroxidase–coupled streptavidin (1:2000; DakoCytomation). Following visualization in a staining solution containing diaminobenzidine and nickel sulfate, sections were incubated overnight at 4°C in guinea pig anti-insulin (1:1000; DakoCytomation). The next day, insulin immuno-reactivity was developed using a horseradish peroxidase–coupled rabbit anti–guinea pig antibody (1:100; DakoCytomation), followed by NovaRed (Vector Laboratories, Burlingame, CA). The double labeling

against Ki-67 and insulin was performed using a similar approach with a rat anti-mouse Ki-67 antibody (1:200; DakoCytomation) as a substitute for the non–β-cocktail, followed by a secondary nonbiotinylated rabbit anti-rat (1:200; Vector Laboratories) and amplification for 30 minutes using a MACH2 goat anti-rabbit horseradish peroxidase polymer (BioCare Medical, Concord, CA). Islet apoptosis was visualized using a rabbit anti-mouse caspase-3 antibody (1:25; Cell Signaling Technology, Danvers, MA).

Stereological estimations were performed on all sections using the newCAST system (Visiopharm, Copenhagen, Denmark) on virtual images scanned on an Aperio ScanScope Scanner. Mass estimates were obtained using a point-counting grid, as described previously (Paulsen et al., 2010). Quantitative estimates of Ki-67–positive cells were performed by manual assessment of double-labeled cells using the auto-disector method for pairing the reference and look-up section (Gundersen et al., 1988). In principle, cells were counted in a reference volume defined by the area of an unbiased counting frame and the distance between the two neighbor sections. The total number of double-labeled cells was finally estimated by multiplying the numerical density with the total reference (β cell) volume.

Statistical Analysis. In vivo data were subjected to relevant statistical analyses using GraphPad Prism (GraphPad Software, La Jolla, CA). Results are presented as mean ± S.E.M. Glucose and insulin area under the curve (AUC) calculations were expressed as total $AUC_{0-240\ minutes}$. Statistical evaluation of the data was carried out using one-way or two-way analysis of variance with appropriate post hoc analysis between control and treatment groups in cases in which statistical significance was established ($P < 0.05$).
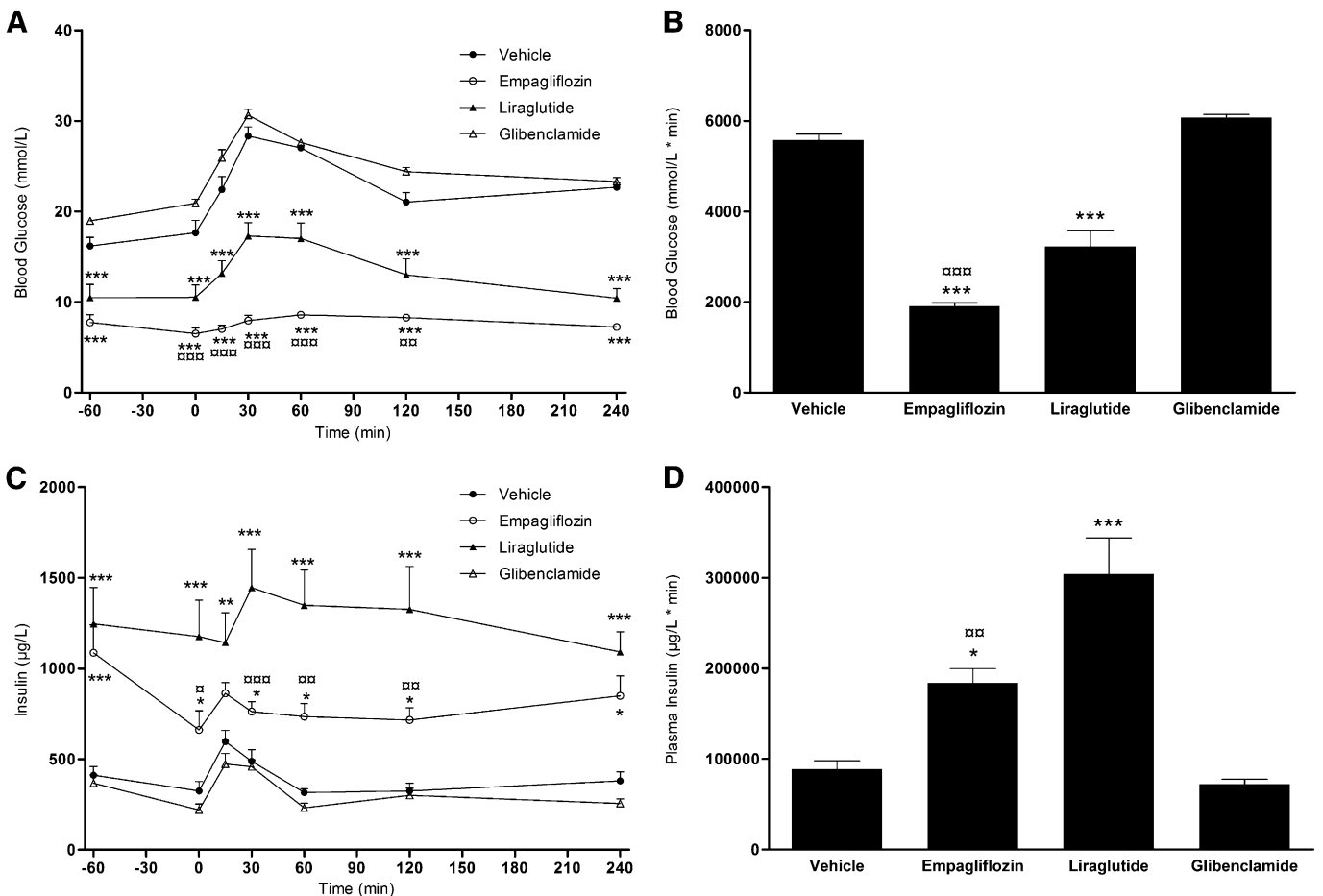
Fig. 2. Effects of empagliflozin, liraglutide, and glibenclamide on glucose (A) and insulin (C) levels during an oral glucose tolerance test following 4 weeks of treatment in male ZDF rats. Corresponding AUC levels are shown in B and D. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ (compared with vehicle control group); $^{\square}P < 0.05$; $^{\square\square}P < 0.01$; $^{\square\square\square}P < 0.001$ (compared with liraglutide).

Results

Blood Glucose, HbA1c, and Insulin Levels. Empagliflozin significantly decreased morning-fed glucose values at all time points (weeks 1–8) when compared with vehicle (Fig. 1A). Similarly, liraglutide had a significant lowering effect on fed glucose levels that attenuated over time and did not attain statistical significance at the end of the 8-week treatment period (Fig. 1A). In correspondence, both empagliflozin and liraglutide significantly lowered circulating HbA1c levels, a measure of long-term glucose regulation, with empagliflozin showing strongest efficacy from treatment week 6 and onward (Fig. 1C). The fed glucose and HbA1c data were supported by determination of weekly insulin levels, showing preserved insulin secretion in the empagliflozin- and liraglutide-treated groups (attenuated over time for the latter), with no effect of glibenclamide on insulin levels (Fig. 1B). Similarly, glibenclamide showed no glycemic effects in the ZDF rats (Fig. 1, A and C).

On study days 28 and 56, rats were subjected to an OGTT (Figs. 2 and 3). As depicted in Fig. 2A, 4 weeks of treatment with empagliflozin or liraglutide significantly reduced baseline fasting blood glucose as well as peak blood glucose level during the OGTT as compared with vehicle-treated animals. The overall reduction in blood glucose values resulted in a statistical significant reduction in AUC for both compounds,

however, with empagliflozin showing the strongest effect (Fig. 2B). Moreover, calculation of the insulin AUC indicated improved insulin release following empagliflozin or liraglutide treatment, as compared with vehicle controls (Fig. 2D). Overall, insulin responsiveness during the OGTT was greater for liraglutide. No effect of glibenclamide was observed.

A second OGTT was performed at the end of the treatment period. A significant decrease in blood glucose was observed for empagliflozin, resulting in significant reduction in glucose AUC (Fig. 3, A and B). Also, liraglutide decreased blood glucose level, but, compared with the first OGTT, the effect on glucose regulation was attenuated. Correspondingly, measurements of plasma insulin during the second OGTT showed significant effects of 8 weeks of empagliflozin treatment on insulin AUC (Fig. 3, C and D). In contrast, the effect of liraglutide on insulin secretion did no longer lead to a significant increase in insulin AUC level (Fig. 3, C and D).

Stereological Assessment of β-Cell Mass. None of the compounds affected total pancreas mass (corrected for fat infiltration and lymph tissue) after 4 weeks of treatment, whereas a slight reduction was observed after 8 weeks of treatment with liraglutide as compared with vehicle controls (Fig. 4A). A histologic assessment of general islet structure indicated that pancreatic islet morphology was affected by the progression of hyperglycemia in control ZDF rats, as demonstrated by the
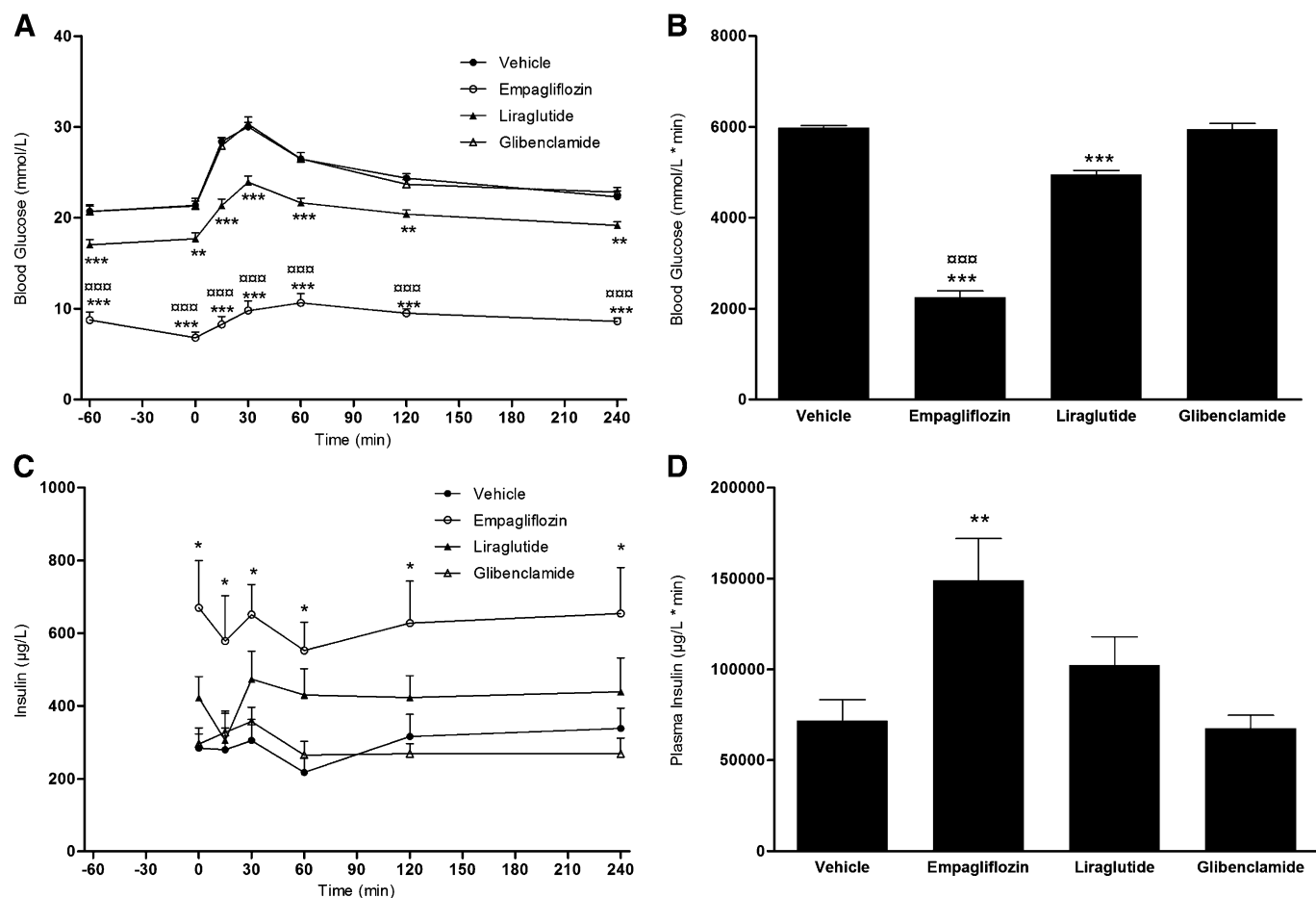
Fig. 3. Effects of empagliflozin, liraglutide, and glibenclamide on glucose (A) and insulin (C) levels during an oral glucose tolerance test following 8 weeks of treatment in male ZDF rats. Corresponding AUC levels are shown in B and D. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ (compared with vehicle control group); ¤¤¤$P < 0.001$ (compared with liraglutide).
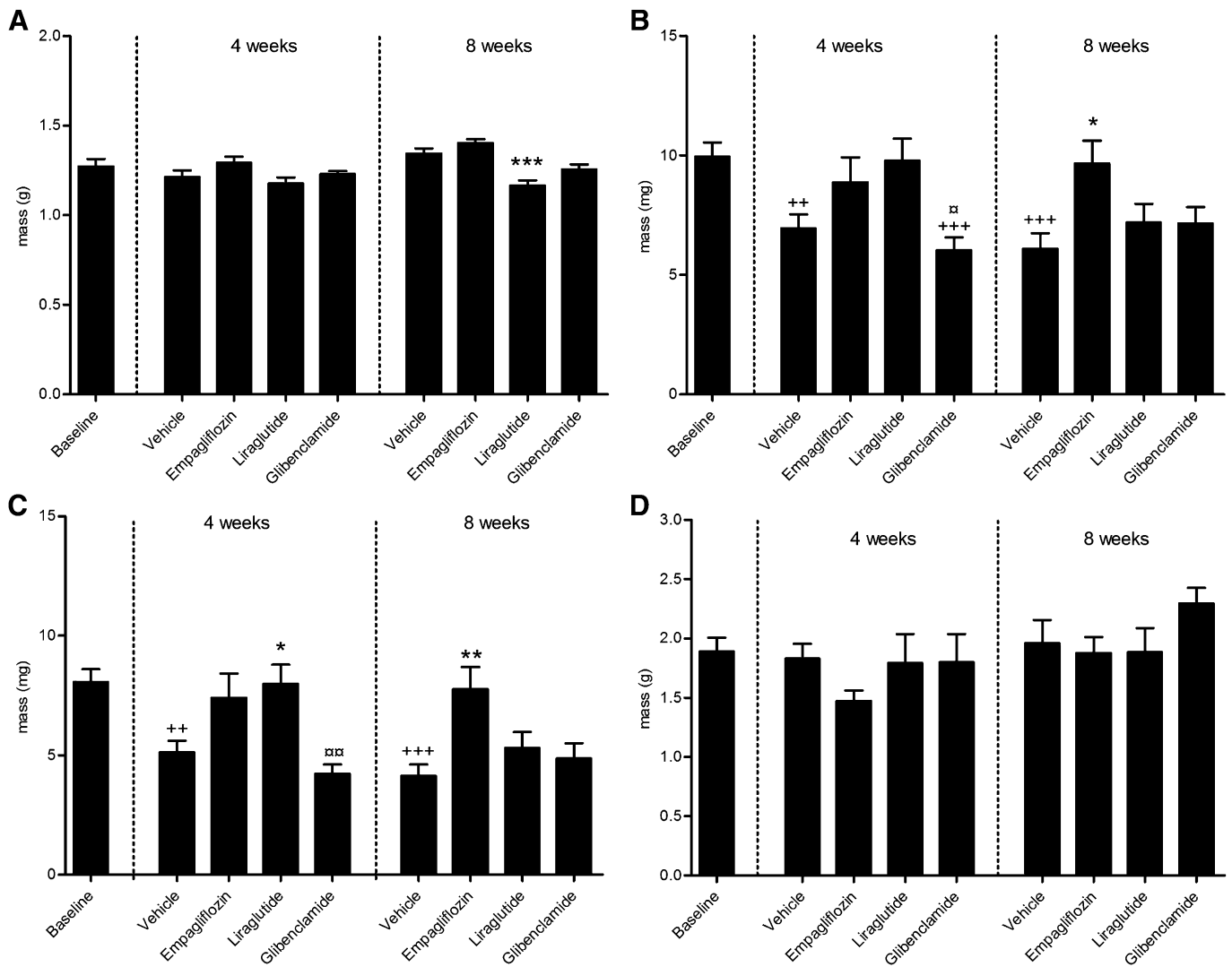
Fig. 4. Effects of empagliflozin, liraglutide, and glibenclamide on pancreas mass (A), islet mass (B), β-cell mass (C), and non–β-cell mass (D) in male ZDF rats. $^{¤}P < 0.05$; $^{¤¤}P < 0.01$; $^{++}P < 0.01$; $^{+++}P < 0.001$ (compared with baseline); $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ (compared with vehicle control group).

appearance of irregular islet architecture (Fig. 5A). A similar pattern was observed in the glibenclamide-treated rats (Fig. 5D), whereas normal islet structure was preserved to some degree in the liraglutide group (Fig. 5C) and more pronounced in empagliflozin-treated rats at both weeks 4 and 8 (Fig. 5B). The quantitative estimation of pancreatic islet mass (composite of β cells and non–β cells) revealed that vehicle-treated ZDF rats had a significant lower total islet mass as compared with the baseline group (Fig. 4B). Empagliflozin treatment led to an improvement in total islet mass, as compared with vehicle controls following 4 weeks of treatment, reaching statistical significance at 8 weeks (Fig. 4B). Liraglutide showed a similar trend at 4 weeks of treatment without evidence of further improvement in total islet mass at 8 weeks of treatment. The higher total islet mass in empagliflozin-treated ZDF rats was reflected by a specific improvement in β-cell mass after both 4 and 8 weeks of treatment compared with the respective vehicle controls, but unchanged as compared with baseline levels (Figs. 4B and 5B). In contrast, liraglutide administration only promoted a significant effect on β-cell mass after 4, but not 8, weeks of treatment (Figs. 4C and 5C). Non–β-cell mass was unaffected in all treatment groups (Fig. 4D).

Proliferating Ki-67–immunoreactive β cells were significantly lower in vehicle control ZDF rats, as compared with baseline levels (Fig. 6A), thus corroborating data on a decreased β-cell mass in these animals. A similar trend was observed in empagliflozin- and liraglutide-treated animals, but without reaching statistically significance compared with baseline (Fig. 6A). No statistical significant effects were observed on caspase-3–immunoreactive apoptotic islet cells (Fig. 6B), although both vehicle and glibenclamide tended to have higher apoptotic islet fractions as compared with baseline levels, as well as to empagliflozin- and liraglutide-treated groups, respectively.

Discussion

The present study characterized the pharmacodynamics of empagliflozin, liraglutide, and glibenclamide on glucose homeostasis and β-cell mass in male ZDF rats. Empagliflozin reduced hyperglycemia and improved insulin sensitivity, as measured by OGTT responsiveness, throughout the study, emphasizing the marked long-term antihyperglycemic efficacy of this specific SGLT-2 inhibitor. Moreover, treatment with empagliflozin led to a sustained β-cell sparing effect preventing islet disruption,
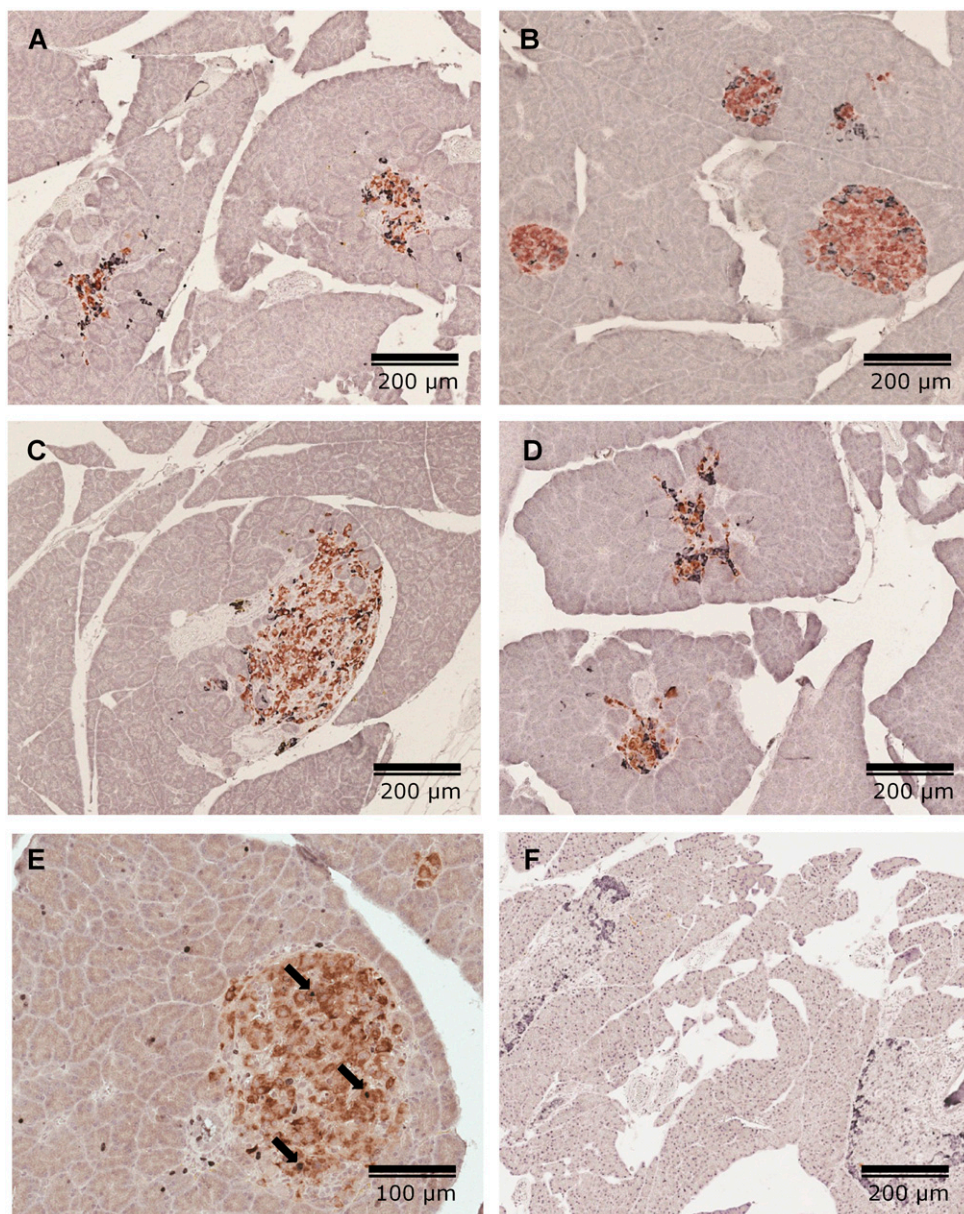
Fig. 5. β cell mass in male ZDF rats treated for 8 weeks with vehicle (A), empagliflozin (B), liraglutide (C), or glibenclamide (D). Ki-67–immunoreactive proliferating β cells (E, arrow denotes Ki-67–positive β cell). F shows a caspase-3–positive islet.

which is a well known characteristic of this animal model of T2DM (Paulsen et al., 2010).

Similarly, liraglutide showed antidiabetic action and improved glucose tolerance during the first weeks of treatment, followed by decreasing efficacy throughout the remainder of the study. In contrast, glibenclamide did not elicit a glucose-lowering effect, which is in line with previous studies reporting lack of antihyperglycemic efficacy of sulfonylureas in male ZDF rats and db/db mice (Biederman et al., 2005; Futamura et al., 2012). ZDF rats are reported to exhibit lowered expression of ATP-sensitive potassium channels, being the target of sulfonylureas (Gyte et al., 2007). Hence, glucose-independent insulin release by glibenclamide may be insufficient, which suggests that the insulinotropic action of glibenclamide was impaired in the ZDF rats as also observed in streptozotocin-induced diabetic rats (Tsuura et al., 1992; Biederman et al., 2005).

The primary goal of this study was to investigate the durability, and not absolute efficacy, of different antidiabetic treatments on glucose homeostasis and how well glycemic changes correlated to pancreatic β-cell mass. Individual compound doses were carefully selected according to standard dose ranges used in diabetic rat models, as reported previously for empagliflozin (Luippold et al., 2012; Thomas et al., 2012), liraglutide (Sturis et al., 2003; Brand et al., 2009; Schwasinger-Schmidt et al., 2013), and glibenclamide (Margolis 1987; Mine et al., 2002; Someya et al., 2009). Because rodents and humans display different pharmacokinetics, it should be noted that the dose ranges applied in preclinical diabetes studies are generally lower than that used in diabetic patients, that is, dosing regimens cannot directly be translated to clinical settings. In addition, a single daily dosing regimen was applied to all treatment groups in the present study; hence, we cannot exclude that individual drug treatment groups would have shown greater glycemic efficacy with repetitive daily dosing or higher dosage to prolong and/or increase drug exposure.
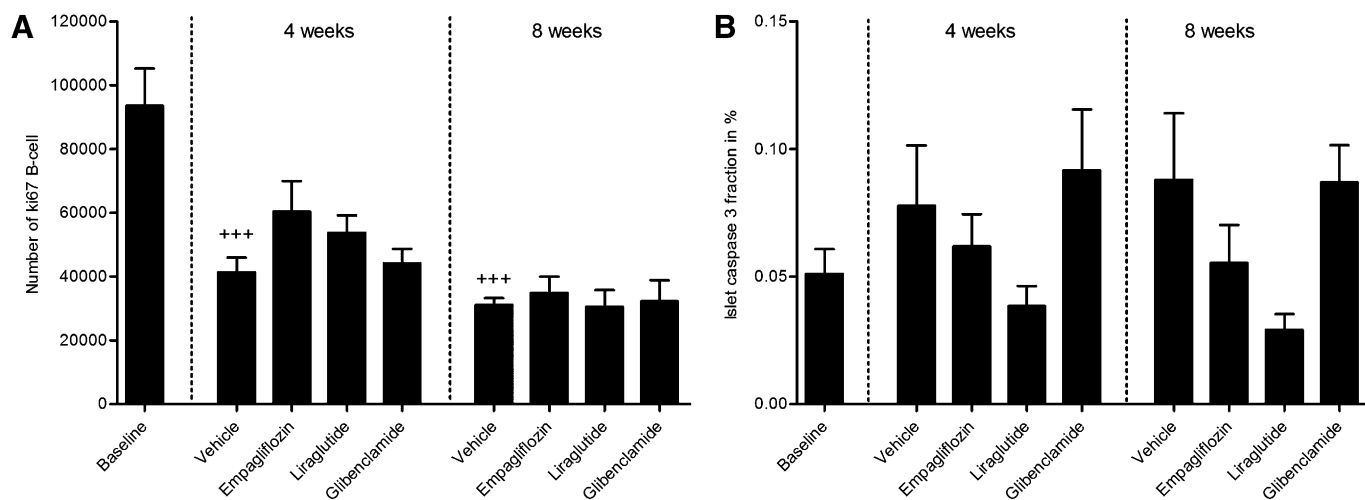
Fig. 6. Effects of empagliflozin, liraglutide, and glibenclamide on Ki-67–immunoreactive proliferating β-cell number (A) and caspase-3–positive apoptotic islet fraction (B), respectively, in male ZDF rats. [+++]$P < 0.001$ (compared with baseline).

Interestingly, empagliflozin treatment led to an improved β-cell mass and function during the whole treatment period despite having no direct effect on β-cell secretory function, as opposed to liraglutide and glibenclamide (Rendell, 2004; Holst, 2007). The present stereological analyses indicated a comparable and significantly higher total islet and β-cell mass in empagliflozin- and liraglutide-treated rats at week 4, however, with only empagliflozin exhibiting a sustained effect following 8 weeks of treatment. Because vehicle controls showed a marked reduction in β-cell mass as compared with baseline, this strongly suggests that improvement in β-cell mass by empagliflozin treatment was caused by preserving β-cell integrity or delaying β-cell decline rather than affecting β-cell proliferation. Hence, pharmacological inhibition of renal glucose reabsorption effectively reduces hyperglycemia with preserved β-cell secretory function and β-cell mass, which strongly suggests that improving glycemic control independent of insulin secretion may lead to β-cell sparing effects of empagliflozin. This interpretation is in good agreement with the hypothesis that glucolipotoxicity and progressive β-cell exhaustion caused by continued increased insulin demand, in combination, may lead to disease progression as a consequence of islet β-cell secretory defects, lowered β-cell proliferative rate, and augmented apoptotic β-cell death (Pick et al., 1998; Prentki and Nolan 2006; Topp et al., 2007; Paulsen et al., 2010). Correspondingly, the present study indicated that vehicle-treated ZDF rats exhibited a significantly lower total islet and absolute β-cell mass with a concomitant significant reduction in the number of Ki-67–positive proliferating β cells as compared with baseline levels. Conversely, β-cell apoptosis levels tended to be increased in vehicle-treated ZDF rats. However, due to the inherently low and variable level of islet caspase-3 immunoreactivity in ZDF rat pancreata, also reported by Futamura et al. (2012), this may most likely explain the lack of consistent inhibitory effects of empagliflozin and liraglutide on β-cell apoptosis. In this respect, we cannot exclude the possibility that β-cell proapoptotic activity might have been higher at an earlier time point, as hyperglycemia in vehicle controls reached a peak level after 3 weeks of treatment. It may thus have been advantageous also to assess caspase-3 levels at earlier time points during treatment.

GLP-1 analogs, including liraglutide, are usually considered to increase β-cell mass in male ZDF rats by specific β-cell receptor–mediated mechanisms. Rather than direct GLP-1 receptor stimulation per se, the present study thus supports the view that improving glycemic control independent of insulin secretory pathways and insulin overproduction constitutes an important mechanism for preventing or slowing down β-cell depletion (Sturis et al., 2003; Vrang et al., 2012). Because empagliflozin directly acts to enhance urinary glucose excretion (Luippold et al., 2012; Thomas et al., 2012), the β-cell sparing effect of empagliflozin strongly supports this notion. Also, it is noteworthy that empagliflozin showed sustained β-cell sparing effects in the ZDF rat, whereas liraglutide was only effective during the first half of treatment period. This observation suggests that GLP-1 receptor stimulation, and hence, concomitant stimulation of β-cell secretory function, did only temporally halt the progression of β-cell failure and disease progress in this rat model of T2DM.

Empagliflozin and other SGLT-2 inhibitors are previously reported to improve glycemic control in preclinical models of T2DM (Arakawa et al., 2001; Fujimori et al., 2009; Liang et al., 2012; Thomas et al., 2012). In addition, a single study demonstrated that 5 weeks of dapagliflozin treatment in moderately hyperglycemic female ZDF rats improved insulin sensitivity in the presence of increased β-cell mass, thus rather representing an early-stage T2DM model (Macdonald et al., 2010). Consequently, the present study is the first to show that SGLT-2 inhibitors, as exemplified by empagliflozin, also evoke β-cell sparing effects in the ZDF rat model of progressed T2DM with features of significantly reduced β-cell mass.

In conclusion, empagliflozin showed pronounced and sustained antihyperglycemic effects in ZDF rats being associated with a slower degradation of β-cell function and β-cell mass. These findings suggest the attractiveness of utilizing SGLT-2 inhibitors to effectively control hyperglycemia by slowing down deteriorating β-cell function in T2DM. Because SGLT-2 inhibitors present a mode of action that does not depend on residual β-cell secretory capacity, this drug class may prove to have broader applicability as compared with insulin-sensitizing and releasing agents currently used in T2DM management. Whether extended long-term treatment with empagliflozin would lead

to preserved β cells and sustained improvement of glucose homeostasis with concomitant delayed disease progression must await further studies.

Authorship Contributions

Participated in research design: Jelsing, Vrang, Klein, Mayoux.
Conducted experiments: H. H. Hansen, C. F. Hansen, G. Hansen.
Performed data analysis: H. H. Hansen, C. F. Hansen, G. Hansen.
Wrote or contributed to the writing of the manuscript: H. H. Hansen, Jelsing, Vrang, Klein, Mark, Mayoux.

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