The role of long non-coding RNAs in the regulation of pancreatic beta cell identity

Maya E. Wilson and Timothy J. Pullen

Department of Diabetes, School of Life Course Sciences, King’s College London, London, U.K.

Correspondence: Timothy J Pullen (timothy.pullen@kcl.ac.uk)

Type 2 diabetes (T2D) is a widespread disease affecting millions in every continental population. Pancreatic β-cells are central to the regulation of circulating glucose, but failure in the maintenance of their mass and/or functional identity leads to T2D. Long non-coding RNAs (lncRNAs) represent a relatively understudied class of transcripts which growing evidence implicates in diabetes pathogenesis. T2D-associated single nucleotide polymorphisms (SNPs) have been identified in lncRNA loci, although these appear to function primarily through regulating β-cell proliferation. In the last decade, over 1100 lncRNAs have been catalogued in islets and the roles of a few have been further investigated, definitively linking them to β-cell function. These studies show that lncRNAs can be developmentally regulated and show highly tissue-specific expression. lncRNAs regulate neighbouring β-cell-specific transcription factor expression, with knockdown or over-expression of lncRNAs impacting a network of other key genes and pathways. Finally, gene expression analysis in studies of diabetic models have uncovered a number of lncRNAs with roles in β-cell function. A deeper understanding of these lncRNA roles in maintaining β-cell identity, and its deterioration, is required to fully appreciate the β-cell molecular network and to advance novel diabetes treatments.

Introduction

Type 2 diabetes (T2D) is a worldwide epidemic affecting over 400 million people [1]. A combination of genetic and environmental risk factors results in a mismatch between the insulin released and the insulin required to correctly regulate blood glucose levels. The resulting sustained hyperglycaemia causes numerous complications including neuropathy, nephropathy and cardiovascular disease which together impose a serious burden of morbidity and mortality.

In healthy individuals, pancreatic β-cells regulate blood glucose levels by the tightly controlled secretion of insulin in response to glucose. To achieve this, β-cells have a highly specialised metabolic system whereby the glucose entering glycolysis is directly proportional to the extracellular concentration. Glucose carbons are then efficiently transported to the mitochondria for oxidative phosphorylation to generate ATP which in turn triggers insulin release [2]. This requires the expression of a group of genes involved in glucose sensing (including SLC2A2 (GLUT2), GCK) and insulin production and processing (including INS, PCSK1). Alternative metabolic pathways which could reduce the efficiency of this process are shut off, including the conversion of pyruvate to lactate and subsequent export from the cell (encoded by LDHA and SLC16A1) [3]. The unique complement of transcription factors essential for maintaining this includes PDX1, MAFA, NEUROD1, NKX6-1 and PAX6 [4]. The ability of β-cells to sense and release sufficient insulin is therefore critically dependent on this pattern of gene expression, which defines mature β-cell identity

In the early stages of T2D, the β-cells’ inability to fully compensate for increased insulin resistance results in impaired glucose tolerance. The increased demand for insulin coupled with the resulting hyperglycaemia progressively damage the β-cells until their decline in function causes overt T2D. Over the last ten years, it has become apparent that a loss of β-cell identity contributes to their
functional decline. This is characterised by the reduced expression of key β-cell transcription factors and the networks of proteins they regulate, as well as increased expression of normally disallowed genes [5]. The maintenance of β-cell identity is critical for glucose homeostasis and understanding how identity is maintained is important to understanding the pathology of T2D.

Long non-coding RNAs (lncRNAs) are transcripts >200 nucleotides long that are typically post-transcriptionally processed but do not encode proteins [6]. They are often enriched in the nucleus and regulate gene expression via a range of mechanisms [6]. lncRNAs differ from some classes of short non-coding RNAs, such as microRNAs (miRNA) which have a more clearly defined mechanism of action. miRNAs are processed from longer transcripts, then associate with AGO2 to target mRNAs for translational inhibition and/or degradation via complementary base pairing [7]. This allows miRNA targets to be predicted bioinformatically. Since lncRNAs are a heterogenous group of transcripts acting via different mechanisms, and the nature of the interactions with other transcripts and proteins are less well known, lncRNA target prediction has generally been much less successful.

While lncRNAs are generally expressed at lower levels than their protein-coding counterparts, their expression patterns are more tissue-specific meaning they are well placed to regulate cell identity [8–10]. In this mini-review, we will discuss the role that lncRNAs play in regulating β-cell identity and the implications for T2D.

**lncRNAs: history, mechanisms and conservation**

In the early 1990s, a small number of RNA molecules were identified which were transcribed by RNA polymerase II, spliced and polyadenylated like mRNAs, but appeared to lack open reading frames. These included H19, expressed from the imprinted Igf2 locus [11] and XIST, from the X chromosome inactivation centre [12]. Deletion experiments identified that Xist was required for X chromosome inactivation [13] and later studies showed that the lncRNA achieves this by recruiting Polycomb Repressive Complex 1 (PRC1) [14]. Airn, expressed from the imprinted Igf2r locus is a long unspliced transcript whose expression is required for silencing three other imprinted genes in this cluster (Igf2r, Slc22a2 and Slc22a3) [15]. These firmly established a role for long non-coding RNAs (lncRNAs) in regulating gene expression.

Technological advances, first in the form of tiling arrays and later high throughput sequencing, identified many more non-protein-coding transcripts. These included HOTAIR, a lncRNA expressed from the HOXC locus on chromosome 12, which interacted with Polycomb Repressive Complex 2 (PRC2) to mediate histone H3 lysine-27 (H3K27) trimethylation and gene silencing at the HOXD locus on chromosome 2 [16]. This demonstrates that lncRNAs can regulate nearby genes both in cis, as well as across much larger distances, in trans.

By 2012, the GENCODE project had identified over 9000 human lncRNA genes [8]. Whether many of these lncRNAs are functional molecules or transcriptional by-products became a point of debate, partly due to their low inter-species conservation and low expression levels [17]. However, investigation of individual lncRNAs has continued to reveal their functional effects and expanded the known mechanisms by which they function. H19 is implicated in tumorigenesis and modulates miRNAs both positively and negatively. The lncRNA is processed to release the growth-suppressing mRNA mir-675 [18]. It also binds to and sequesters several other miRNAs, thus acting as a miRNA ‘sponge’ to down-regulate their action [19]. HOTAIR has a range of trans inhibitory functions spanning the nucleus and cytoplasm, including recruitment of histone demethylases for gene suppression and ubiquitin ligase recruitment for protein degradation [20]. These mechanisms are non-mutually exclusive as lncRNAs may have diverse cellular roles [6].

While their sequences are less conserved than protein-coding sequences, lncRNAs are significantly more conserved than random intergenic regions [21]. lncRNA function is facilitated by the ability to form secondary structures that enables their interaction with nucleic acids and proteins. lncRNAs also interact with miRNAs to alter their stability or miRNAs to sequester them based on nucleotide sequence complementarity. In both cases, lncRNA functionality is less dependent on precise nucleotide sequence as compared with amino acid sequence determining protein function [22]. Furthermore, promoter regions of lncRNA genes have been shown to contain transcripion factor binding sites and generally contain similar levels of sequence homology to protein-coding gene promoters [8]. These regions can be used to identify orthologous lncRNA genes between species [23].

**lncRNAs genetically associated with type 2 diabetes**

The first clear evidence of lncRNA involvement in β-cell function came from genetic association. It became apparent from early genome-wide association studies (GWAS) that most T2D-associated SNPs occurred in non-protein-coding regions of the genome [24]. While variants in protein-coding regions often result in an
amino acid substitution or protein termination, the molecular effect of variants in non-protein-coding regions can be harder to determine. Several T2D-associated SNPs were identified in loci expressing lncRNAs including CDKN2B-AS1 (ANRIL) [25–27] and KCNQ1OT1 [28,29]. Indeed, the CDKN2B-AS1 locus cropped up in so many diverse studies of susceptibility to cancer and metabolic disease that it was described as a ‘GWAS hotspot’ [30].

The CDKN2A/B locus contains two protein-coding genes encoding the three well-studied cyclin-dependent kinase inhibitors p16INK4A, p15INK4B and p14ARF, and a lncRNA gene CDKN2B-AS1 (Figure 1A). The importance of this locus to the regulation of cellular proliferation explains the links to cancer and suggests dysregulation of β-cell proliferation may cause the increased T2D susceptibility. However, disentangling the effects of the various SNPs in this complex locus is not straightforward. The T2D-associated SNPs fall within the

---

**Figure 1. Genomic positioning and directional regulation of lncRNA genes in relation to nearby β-cell genes.**

(A) CDKN2B-AS1 (ANRIL) mechanism of action remains unclear, as it has potential PRC1/2 binding potential, but is also positively correlated with CDKN2A/B. (B) KCNQ1OT1 negatively regulates its neighbour CDKN1C to maintain β-cell proliferation. (C) PLUTO positively regulates PDX1 by enhancing its promoter-enhancer cluster contacts. (D) βlinc1 has wide-spread cis effects on local gene expression to maintain β-cell function, positively regulating Nkx2-2, Neurog3, Sox9, Nkx6-1, NeuroD1, Pax6, Pax4, Arx and MafB. (E) HI-LNC25 has a trans-acting effect on GLIS3, located on another chromosome. (F) ROIT neighbours ESRP2 but has trans effects on Nkx6-1.
IncRNA gene and, at least in peripheral blood samples, are more closely associated with the IncRNA expression than the protein-coding genes [30,31]. However, the regulation of this locus differs between cell types and the difficulty in accessing human islet tissue and the lack of conserved IncRNAs in the mouse genome means relatively few studies have directly investigated the function of CDKN2B-AS1 in β-cells. The best evidence for a role in β-cells is that T2D-associated SNPs increased expression of CDKN2B-AS1, and while they did not affect insulin secretion, they were associated with impaired β-cell proliferation [32]. CDKN2B-AS1 can down-regulate neighbouring genes by recruiting PRC1 and PRC2 components [33]. However, at least in islet samples, the expression of CDKN2B-AS1 and the transcripts encoding p14 and p16 are strongly positively correlated implying that coregulated expression outweighs any inhibition of the other genes by the IncRNA [32]. CDKN2B-AS1 has also been shown to regulate gene expression on other chromosomes, including down-regulating KLF2 to regulate apoptosis in cancers [34,35]. Therefore, while there is evidence for a role for CDKN2B-AS1 in regulating β-cell proliferation and diabetes susceptibility, the mechanism by which it achieves this remains unclear. The possibility that SNPs at this locus affect diabetes susceptibility through effects in other tissues can also not be excluded [36].

The KCNQ1 locus is another region that is central to the regulation of cellular proliferation and associated with T2D susceptibility. It is an imprinted region which expresses an IncRNA, KCNQ1OT1, orientated antisense to the KCNQ1 gene which encodes a voltage-gated potassium channel subunit (Figure 1B). KCNQ1 knockout in mice and knockdown in human islets appears to have no effect on glucose tolerance and insulin secretion, making it very unlikely to affect T2D susceptibility [37]. The locus also contains the CDKN1C gene encoding P57KIP2, an inhibitor of cyclin-dependent kinases and known regulator of β-cell proliferation [38]. KCNQ1OT1 interacts with G9a and PRC2 to mediate histone methylation and down-regulation of CDKN1C and SLC22A18 [39]. The SNPs at this locus are hypothesised to mediate diabetes susceptibility by disrupting the expression of KCNQ1OT1, altering the imprinting within this gene cluster and leading to the overexpression of CDKN1C and impaired β-cell proliferation [37].

Another imprinted locus regulating cellular proliferation is the H19/IGF2 locus, which both expresses an IncRNA and has more recently been genetically associated with T2D susceptibility [40]. H19 is an intergenic IncRNA that has been implicated in β-cell mass expansion during neonatal development. Adult β-cells have limited proliferative capacity as compared with neonatal β-cells which are characterised by ~300 times greater H19 expression. Silencing H19 in neonatal rat β-cells reduced their proliferation, whereas overexpression increased proliferation of adult β-cells via the PI3K–Akt pathway. H19 was also significantly increased in ob/ob and db/db diabetic mouse models with increased β-cell mass, supporting the role of H19 in β-cell mass expansion [41].

It is interesting to note that these IncRNAs appear to influence T2D susceptibility through impaired proliferation causing an insufficiency in β-cell mass rather than via a loss of β-cell identity. Since the early GWA studies, many more β-cell IncRNAs have been identified and in some cases functionally characterised. The most comprehensive catalogue of IncRNAs expressed in human β-cells identified 1128, many of which were conserved in mouse [21]. This catalogue has proved a vital resource for subsequent studies.

**β-cell transcription factors and IncRNAs**

The key β-cell transcription factors, including PDX1, MAFA, NEUROD1 and PAX6, are responsible for regulating the expression of genes involved in insulin synthesis and GSIS, and are thus central to regulating β-cell identity [4]. Given the potential for IncRNAs to regulate expression of nearby genes, β-cell IncRNAs located near β-cell transcription factor genes have often been selected to identify novel regulators of β-cell identity. These studies have provided good evidence that IncRNAs and β-cell transcription factors can be co-regulated such that if the expression of a IncRNA is altered, its neighbouring protein-coding counterpart will also be altered [42]. For example, expression of the IncRNA PLUTO is highly correlated with its neighbour PDX1 (Figure 1C). Knockdown of PLUTO reduced PDX1 expression and resulted in a similar transcriptional profile to that of a PDX1 knockdown. Chromatin conformation capture assays revealed that PLUTO positively regulates PDX1 by enhancing contacts between its promoter and an upstream enhancer cluster [42] demonstrating that PLUTO regulates gene expression by modifying the 3D chromosomal structure.

βlinc1 is another IncRNA which appears to regulate a nearby transcription factor gene. The βlinc1 gene is located between Nkx2-2 and PAX1 in humans and mice (Figure 1D) and its expression is highly enriched in β-cells [43]. βlinc1 knockdown in mouse insulinoma cells (MIN6) resulted in down-regulation of Nkx2-2, indicating the IncRNA is a positive regulator of Nkx2-2 expression, although the mechanism is unknown. βlinc1
knockout mice were glucose intolerant due to an alteration in the ratio of insulin- and somatostatin-producing cells that originated during pancreatic development, as evidence for the role of \( \beta \)-linc1 in maintaining \( \beta \)-cell identity. Both hetero- and homozygous \( \beta \)-linc1 knockouts resulted in dysregulated expression of mature functional \( \beta \)-cell genes including Neurog3, Sox9, Nkx6-1, NeuroD1, Pax6, Pax4, Arx and MafB. Many dysregulated genes were found within a \( \sim \)55Mb radius of \( \beta \)-linc1 on chromosome 1, indicating a cis-acting role of \( \beta \)-linc1 in \( \beta \)-cell development and identity [43].

In contrast with the previous two examples, the PAX6 locus contains a lncRNA gene which appears to be a negative regulator of \( \beta \)-cell identity. Pax6os1 (mouse)/PAX6-AS1 (human) is orientated antisense to PAX6 and displays increased expression in islets from donors with T2D and diabetic mouse models. Pax6os1 knockdown in MN6 and CRISPR-mediated knock out in a human \( \beta \)-cell line (EndoC-\( \beta \)-H1) resulted in increased expression of PAX6 and several other \( \beta \)-cell genes including INS and SLC2A2 (GLUT2) [44]. However, these effects were not observed in a Pax6os1 null mouse, possibly because the deletion of the lncRNA promoter and first exon also removed regulatory sequences for Pax6.

While many lncRNAs expressed from \( \beta \)-cell transcription factor loci are involved in regulating the expression of nearby genes, some appear to regulate gene expression on other chromosomes. HI-LNC25 (LINC01370) neighbours MAFB on chromosome 20 (Figure 1E), yet knockdown of this lncRNA produced no effect on MAFB expression while decreasing expression of GLIS3, located on chromosome 9 [21]. GLIS3 encodes a transcription factor important for both \( \beta \)-cell development [45] and insulin transcription [46]. While HI-LNC25 depletion had no significant effect on GSIS, it was enriched in human islets compared with embryonic pancreas suggesting a developmental role for this lncRNA [21]. Similarly, lncRNA ROIT (1810019D21Rik) has a role in glucose homeostasis and insulin transcription. ROIT regulates expression of the neighbouring ESRP2 gene (Figure 1F), but also positively regulates NKX6-1 in trans by inhibiting DNMT3A-mediated DNA methylation of its promoter [47]. It is therefore clear that lncRNAs expressed from transcription factor loci in \( \beta \)-cells are involved in both local and distant regulation of gene expression underlying \( \beta \)-cell identity.

**lncRNA expression levels in diabetic models**

Expression changes in animal models of diabetes have been used to identify lncRNAs regulating \( \beta \)-cell identity. \( \beta \)-linc2 and \( \beta \)-linc3 are highly islet- and \( \beta \)-cell-enriched, with \( \beta \)-linc2 being significantly up-regulated in high-fat diet and \( db/db \) mice, whereas \( \beta \)-linc3 tended to be down-regulated in these groups [48]. \( \beta \)-linc3 was also significantly reduced in islet donors with T2D and negatively correlated with both BMI and HbA1c. \( \beta \)-linc2 overexpression and \( \beta \)-linc3 down-regulation both increased apoptosis of MIN6 cells and primary mouse islets yet had no clear effect on insulin synthesis or secretion. Preliminary investigation into \( \beta \)-linc2 indicated it may stimulate apoptosis by shuttling NF-\( \kappa \)-B into the nucleus [48].

Two further lncRNAs, Tug1 and Meg3, were singled out because of their glucose-regulated expression. Tug1 is pancreas-enriched and its expression was reduced in two \( \beta \)-cell lines exposed to increasing glucose concentrations [49]. In vivo siRNA-knockdown of Tug1 resulted in reduced cell proliferation, increased apoptotic caspases and decreased anti-apoptotic Bcl2 in vitro and in vivo. This was accompanied by reduced GSIS, higher blood glucose levels and decreased expression of \( \beta \)-cell genes Pdx1, NeuroD1, MafA and Slc2a2 [49], clearly demonstrating that Tug1 reinforces \( \beta \)-cell identity.

In contrast with Tug1, Meg3 was up-regulated in response to increasing concentrations of glucose in MIN6 cells, correlating well with levels of Ins2 [50]. siRNA knockdown of Meg3 led to impaired glucose tolerance, decreased insulin content and insulin secretion during a glucose challenge. Dysregulated Meg3 also resulted in lowered Pdx1 and MafA expression but had no significant effect on NeuroD1 or Nkx6-1. Like Tug1, Meg3 knockdown inhibited cell growth due to increased apoptosis [50]. Mechanistically, Meg3 has been shown to interact with PRC2 and form RNA-DNA triplex structures at specific genomic sites, facilitating chromatin silencing in a breast carcinoma cell line [51]. Similarly, RNA immunoprecipitation in MIN6 cells found Meg3 was associated with the PRC2 component, EZH2. Together they increase MafA expression by down-regulating three repressive transcription factors Rad21, SMC3 and Sin3a [52]. These studies have made use of mouse models and cell lines to emphasise the functions of key lncRNAs in \( \beta \)-cell transcriptional regulation, however further studies highlighting the roles of these lncRNAs in humans is required.

The links between lncRNAs and T2D have also been investigated by identifying changes in circulating lncRNAs associated with diabetes. As an example, lncRNA-p3134 was significantly increased in serum and specifically enriched in the circulating exosomes of humans with diabetes and mouse models. Overexpression of this lncRNA resulted in increased expression of \( \beta \)-cell-specific genes such as Pdx1, MafA and Slc2a2, leading
to increased glucose-stimulated insulin secretion (GSIS) via activation of the PI3K/Akt/mTOR pathway. IncRNA-p3134 overexpression also provided a protective effect against glucotoxicity-mediated apoptosis, thereby maintaining β-cell mass for a sufficient insulin secretory response [53].

In contrast with IncRNA-p3134, the IncRNA GAS5 shows significantly reduced expression levels that correlate with HbA1c and T2D in humans, highlighting its potential use as a serum biomarker [54,55]. siRNA knockdown of Gas5 in MIN6 cells and mouse islets impaired expression of insulin mRNA and GSIS, as well as reducing expression of β-cell genes Pdx1, Mafa, NeuroD1, and Slc2a2. In contrast, Gas5 overexpression improved cell viability, GSIS, and insulin content [54]. Further investigation into Gas5 demonstrated a role in miRNA sequestration, as expression levels of three miRNAs with roles in insulin signalling, secretion and resistance (miR-29a-3p, miR-96-3p, and miR-208a-3p) were inversely correlated with Gas5 levels during Gas5 overexpression and knockdown. Gas5 has conserved binding sites for these miRNAs and pulldown assays confirmed their interaction as evidence for its mechanism for maintaining β-cell identity [54]. Another mechanism by which Gas5 can act is by repressing the glucocorticoid receptor (GR) through competitively inhibiting the DNA binding motif [56]. Glucocorticoids (GC) inhibit β-cell function and steroid-induced diabetes is a common side effect of GC treatment. This may be partially mediated by GAS5, since GC treatment decreases GAS5 expression in EndoC-βH1 cells, and both GC treatment and GAS5 knockdown inhibit GSIS. GC-impaired GSIS was rescued by the overexpression of the active segment of Gas5 called the hormone response element mimic (HREM), raising the possibility that RNA-based therapeutics could be used to alleviate steroid-induced diabetes [57].

DANCR is another IncRNA which acts by sequestering miRNAs, in this case miR-33a-5p which is disproportionately up-regulated in the serum of patients with gestational diabetes. DANCR overexpression can rescue the compromising phenotype created by miR-33a-5p overexpression, restoring cell proliferation and insulin output in an INS-1 insulinoma model [58]. Further studies are likely to uncover more IncRNAs that exhibit their function by miRNA sequestration.

**Conclusion**

Pancreatic β-cells express over a thousand IncRNAs, although only a small percentage of these have been studied in detail. It is clear from the few which have been investigated that β-cell-specific IncRNAs are regulators of β-cell transcription factor expression, both in cis and trans, thus regulating β-cell identity. Beyond the transcription factors, IncRNAs have also been shown to regulate expression of other genes, affecting insulin production and secretion, glucose sensitivity, maturity and proliferation.

In a few cases, the molecular mechanisms by which the IncRNAs exert their effects have been revealed, but in many cases they have not. The examples discussed here demonstrate that IncRNAs regulate β-cell gene expression through both chromatin and DNA methylation, affecting chromosomal conformation and both positive and negative effects on miRNA actions. More detailed mechanistic studies of the remaining IncRNAs and distinguishing between direct and indirect gene expression changes will be important to fully understand how IncRNAs regulate β-cell identity and function.

The highly cell-type specific expression of many IncRNAs coupled with their role in regulating β-cell identity raises the possibility that they could be therapeutically targeted to reinforce β-cell function in T2D. IncRNAs also show potential as circulating biomarkers of β-cell dysfunction during T2D.

**Perspectives**

- **Importance of the Field:** Pancreatic β-cell identity is vital for maintaining the patterns of gene expression necessary for correctly regulated insulin secretion. The loss of β-cell identity contributes to type 2 diabetes (T2D) progression, so understanding how cell identity is regulated is central to restoring β-cell function as a new approach to treat the disease. Long non-coding RNAs (lncRNAs) are highly cell-type specific transcripts involved in the regulation of gene expression in a range of tissues.

- **Summary of Current Thinking:** β-cell specific IncRNAs have been shown to regulate key transcription factors and a variety of other genes underlying β-cell identity and function. IncRNA loci have also been genetically associated with T2D susceptibility, although this appears to be through regulating β-cell mass rather than identity.
Comment on Future Directions: Given their important role in regulating β-cell identity, the next steps will be to gain greater mechanistic insight into how these lncRNAs function and whether they can be therapeutically manipulated to reinforce β-cell function in T2D. The hundreds of uncharcterised lncRNAs expressed in β-cells may also contain other important players in the complex networks which regulate cell identity.

Competing Interests
The authors declare that there are no competing interests associated with the manuscript.

Funding
This work was supported by the joint King’s College London and Technische Universität Dresden IRTG programme and by Diabetes UK (grant number 19/0006062).

Open Access
Open access for this article was enabled by the participation of King’s College London in an all-inclusive Read & Publish pilot with Portland Press and the Biochemical Society under a transformative agreement with JISC.

Author Contributions
M.E.W. and T.J.P. contributed to the conceptualization, writing — original draft and writing — review and editing of this review.

Abbreviations
GC, glucocorticoids; GSIS, glucose-stimulated insulin secretion; GWAS, genome-wide association studies; PRC1, Polycomb Repressive Complex 1; PRC2, Polycomb Repressive Complex 2; SNPs, single nucleotide polymorphisms.

References
2160

© 2021 The Author(s). This is an open access article published by Portland Press Limited on behalf of the Biochemical Society and distributed under the Creative Commons Attribution License 4.0 (CC BY).


© 2021 The Author(s). This is an open access article published by Portland Press Limited on behalf of the Biochemical Society and distributed under the Creative Commons Attribution License 4.0 (CC BY).