Combined sections History of Insulin (D1)

Title: Insulin Centennial:

Milestones influencing the development of insulin preparations since 1922.

Authors: David R Owens MD[1], Louis Monnier MD[2], Antonio Ceriello[3], Geremia B Bolli MD[4]

[1]University of Swansea Medical School, Wales, UK

[2]Institute of Clinical Research, University of Montpellier, France

[3]IRCCS MultiMedica, Milan, Italy

[4]Department of Medicine, University of Perugia, Italy

Corresponding Author: D R Owens, Diabetes Research Unit Cymru, University of Swansea Medical School, Singleton Park, Swansea SA2 8PP, Wales, United Kingdom. E-mail D.R.Owens@swansea.ac.uk

ORCID number: https:// orcid.org/0000-0003-1002-1238

Abstract

During 1921-22, a team effort by Banting, Macleod, Collip and Best isolated and purified insulin and demonstrated its life-giving properties, giving rise to the birth of insulin therapy.

In the early years (1922-50s), priorities revolved around the manufacture of insulin to meet demand, improving purity to avoid allergic reactions, establishing insulin standards and increasing its duration of action to avoid multiple daily injections. Shortly after the emergence of insulin, Joslin and Allen advocated the need to achieve and maintain good glycaemic control to realise its full potential. Although this view was opposed by some during a dark period in the

history of insulin, it was subsequently some sixty years later endorsed by the DCCT and UKPDS trials. Major scientific advances by the Nobel Laureates Sanger, Hodgkin, Yalow and Gilbert and also by Steiner have revolutionised the understanding of diabetes and facilitated major advances in insulin therapy.

The more recent advent of recombinant technology over the last 40 years has provided the potential for unlimited source of insulin, and the ability to generate various insulin 'analogues', in an attempt to better replicate normal insulin secretory patterns. The emerging biosimilars now provide the opportunity to improve availability at a lower cost.

Word Count 200

1.Introduction

Before the availability of insulin, clinical management of diabetes was grim and frustrating for people with diabetes and carers alike, involved prolonged fasting and severe carbohydrate restriction (starvation). As noted by Allen in 1917, this was "in the hope of a more positive and powerful therapy to come" (1).

Among attempts to extract insulin at the beginning of the last century, the most encouraging were reported by George Ludwig Zülzer (1908), Ernest Lyman Scott (1912), Nicholae Paulescu (1916-23) and Israel Kleiner (1919) (Figure 1). Although their extracts successfully achieved a reduction in glycosuria, the accompanying adverse reactions were blamed on contaminants as none had

recorded blood sugar levels during such events. Other obstacles, including the Great War in Europe, and the lack of support denied these researchers from reaching the ultimate goal.

Figure 1

The story of this epoch-making discovery of insulin and its introduction into the management of diabetes, has been retold many times, most comprehensively by the eminent historian Michael Bliss (3,4).

This most significant advance in the field of endocrinology in the 20th century, involved four key contributors from Toronto, Canada. Frederick Grant Banting, an orthopaedic surgeon, who can be considered as the 'instigator' of this project, his mentor, John James Rickard Macleod, Professor of Physiology and head of the laboratory in which the research took place, the chemist James Bertram Collip and finally the medical student Charles Herbert Best (Figure 2).

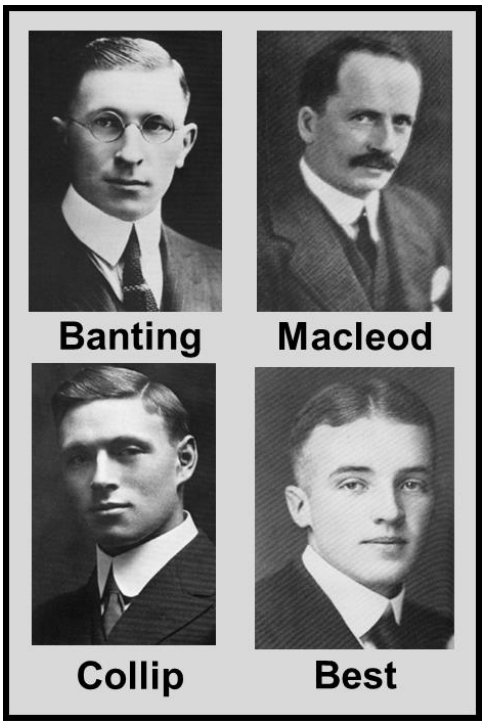

Figure 2

In 1923, the Nobel prize in Medicine or Physiology was awarded to Banting and Macleod “for the discovery of insulin”. Although insulin had been discovered by earlier researchers (some mentioned above), the contribution of Banting and Macleod embodied not only the isolation and purification of insulin, but also its initial application to people with diabetes whose lives were otherwise doomed (5). A more appropriate Nobel Prize citation could be “for the birth of insulin therapy.” Unfortunately, discourse between the four Toronto researchers led to Banting sharing his stipend with Best, and Macleod with Collip. Macleod was sceptical at the outset, predicting to Banting in 1920 that the outcome would be “a negative result of great physiological importance”. However, his contribution, as a leading physiologist of his time, in the planning and execution of the research was fundamental (6,7,8). He provided vital guidance to the two young inexperienced, although energetic researchers, throughout the challenging times ahead, thereby ensuring with Collip’s vital contribution that the goal of achieving a life-sustaining therapy for people with diabetes, was eventually realised.

The availability of insulin in 1922 heralded the end of an earlier era of disappointed expectations only to be confronted by new challenges. In 1928 Joslin wrote that “there has never been anything discovered as valuable for the diabetic as insulin, but diabetes, though subdued, is not yet conquered” (9).

This review summarises the views of the authors on some of the key milestones that influenced the advancements in the *galenics* and application of insulin during the last 100 years, broadly divided into the period before and after the advent of recombinant DNA technology in 1980 to advance insulin therapy.

2. The pre-recombinant DNA period 1922-1980

2.1 Production, Purification, Potency of insulin and Prolonging its duration of action

2.1.1 Production

The administration of a 'thick brown extract' of bovine pancreas (Macleod's serum) by subcutaneous injection to the boy Leonard Thompson on January 11, 1922 (10) heralded the end of the 'Frustration Era' in the management of type 1 diabetes (T1DM) (11). It should be noted that only a moderate fall in blood sugar was observed with the first dose of the pancreatic extract, and was complicated by the development of a sterile abscess at one of the injection sites. When treatment was resumed on January 23 using a purer extract (Collip's serum), blood sugar lowering was much improved, and a favourable clinical outcome was reported. This was quickly replicated in a number of other children at the same hospital (12). Future challenges following the initial excitement quickly ensued with a need to expand insulin production, improve purity, prolong the insulin action, improve insulin delivery and develop techniques for adequate monitoring.

To expand production, insulin manufacture was relocated to the Connaught Medical Research Laboratories in Toronto with Collip in charge, and a dedicated Insulin Committee formed to oversee future developments. The introduction of isoelectric precipitation to the extraction process by George Walden a chemist at the Eli Lilly

Pharmaceutical Company greatly improved yield and purity. Along with enhanced supplies of insulin came notable successes. Joslin acknowledged in 1923 that “The contribution of Banting and Best to the treatment of diabetes is greater than I ever expected to witness” (9).

In March 1923, Eli Lilly began to produce insulin commercially, followed shortly thereafter by other approved manufacturers in the USA, Europe and Australia.

The early commercial production of crude insulin produced an amorphous precipitate that included only 50-60% insulin (13). Additional purification steps became necessary due to the high frequency of local allergic reactions (14). In 1926, Abel and colleagues were first to crystallise insulin, (15), with zinc added to the process in order to further enhance its purity (16). The introduction of multiple recrystallisation steps subsequently achieved a purity of 80-90%, which substantially reduced the risk of allergic reactions (17). Insulin neutralising antibodies were nevertheless noted on radio-immunoassay (RIA) in all patients receiving re-crystallised insulin (18). The availability of newer analytical techniques (e.g., partition and anion-exchange chromatography, disc electrophoresis and gel filtration) highlighted the considerable heterogeneity in re-crystallised insulin. The identified impurities were found to be primarily responsible for generating anti-insulin antibodies (19,20). Consequently, multiple chromatographic steps were introduced to achieve highly purified insulins (e.g., monocomponent (MC) insulin that contained contaminants at or below detection limits (21).

In 1928, insulin was discovered to be a protein, with its primary structure revealed in the 1950s, by Frederick Sanger (Figure 3a) and colleagues who delineated the amino acids sequence from several animal species (22). In 1960, the sequence of human insulin was reported (23) followed by several attempts at its synthesis. During the 1960s, the peptide synthesis of sheep and bovine insulin was achieved, followed by the total chemical synthesis of human insulin (24). Although, this was biologically equivalent to the natural hormone, it was considered to be too costly as a source for clinical use.

Frederick Sanger

Figure 3a

Subsequently, efforts to enzymatically substitute the single amino-acid difference between porcine ($B30^{Ala}$) and human($B30^{Thr}$) insulin were made in the 1970's. Although Obermeier and Geiger succeeded in achieving this in 1976, their yield was too low (<10%) (25). The discovery that enzymes in mixtures of water and organic solvents shifts peptide bond equilibria towards synthesis (26) made transpeptidation of porcine to human insulin possible (27). In 1987, Markussen used trypsin with a large excess of threonine ester to replace the $B30^{Ala}$ residue, followed by cleavage of the $B30^{Thr}$ ester group to produce human insulin. This became become the first naturally occurring protein to be manufactured by semi-synthesis (28), and highly purified (MC) semi-synthetic human insulin became available in 1980.

These highly purified insulins showed lower insulin antibody titres in most patients compared with recrystallised insulin (29), fewer allergic reactions, rarely lipodystrophy, and generally lower insulin dosage and improved metabolic control. Semi-synthetic human

insulin was less immunogenic than bovine or porcine insulin at equivalent purity (30), although glycaemic control remained similar.

2.1.2 Potency/standardisation of animal derived insulin: The unit of insulin

Defining insulin potency and standardisation was a process that evolved over more than 60 years. Banting and colleagues initially showed that their extract was effective in normal rabbits, providing a ready source to define potency (31). The Insulin Committee proposed that a unit of insulin should be defined as "that amount of insulin capable of lowering the blood sugar to the convulsive level (blood sugar ≤45 mg/ml) within three hours in rabbits of approximately 2kg in weight having been starved for 24 hours" (32). In 1922, August Krogh introduced the 'mouse unit' as it was more convenient and economical. In view of the associated variability of the bioassays causing potency discrepancies between insulin batches, the British pharmacologist and physiologist Sir Henry Hallett Dale insisted that a reference sample was necessary. Consequently, in Geneva (1925) at the International Standards Conference the first International Standard (IS) of crystalline bovine insulin was assigned a potency of 8 units (U) of insulin/mg. With the rapid advancements in purification technology, a second IS, was introduced in 1935, with a higher potency of 22 IU/mg. The third IS in 1952, had a potency of 24.5 IU/mg with the fourth in 1958 having a designated potency 24.0 IU/mg based on recrystallised bovine (52%) and porcine (48%) insulin. With the availability of highly purified insulin in the 1980s, and the associated time dependent variation in hypoglycaemia between bovine, porcine and human insulin, meant that species-specific insulin standards were necessary. Consequently, the World Health Organisation in 1986, assigned *in vivo* potencies for human (semi-synthetic), porcine and bovine insulin of 26.0, 26.0 and 25.7 IU/mg, respectively. In its anhydrous form, the potency of the human

insulin standard was 28.8 IU/mg. Based on quantitative amino acid analysis of human insulin, 1 conventional IU is equivalent to 6.00 nmol SI units.

In 1923, the concentration of insulin increased from 10 to 20 IU/ml, reaching 40 IU/ml in 1924 and 80 IU/ml in 1925 (33). Having such different strengths led to confusion and resulting dosage errors, only alleviated by adoption of a single strength, 100 IU/ml, in the 1970s and 1980s. Higher strengths (200 - 500 IU/ml) have also become available for use in special circumstances.

Henry Dale's contribution to the standardization of insulin, during the years 1923-1967, was an essential prerequisite in ensuring uniformity across the different manufacturers with patient safety in mind (34,35).

2.1.3 Development of protracted-acting insulins (1930s-1950s)

The need for multiple daily injections of the original soluble/regular insulin prompted efforts to delay its subcutaneous absorption, thereby extending its glucose lowering effect, especially during the nocturnal period. Attempts to mix insulin with gum arabic solutions, oil suspensions, lecithin emulsions or vasoconstrictor substances, met with little or no success due to pain on injection, variability in absorption and/or poor stability (36).

Greater success occurred when insulin was complexed with other proteins (37) or metals ions were added to the insulin solution (38). In 1936, Hans Christian Hagedorn, combined insulin with protamine (extracted from the sperm of the trout, *Salmo iridius*), which reduced its solubility at neutral pH (37). A colleague, Charles Krayenbuhl discovered the optimal relationship between insulin and protamine, where no excess of either exist in solution after precipitation (i.e. in 'isophane'- stoichiometric proportions). The original unstable

protamine insulate suspension required a phosphate buffer to be added prior to administration. Although its protracted action was evident (39) controlling postprandial hyperglycaemia required additional soluble insulin. The stability issue was solved by Scott and Fisher in 1936 using surplus protamine with a small amount of added zinc, producing protamine zinc insulin (PZI) (40). PZI possessed a prolonged action but was complicated by occasional episodes of severe hypoglycaemia that occurred without warning (41). Separate injections of regular insulin before meals were also required. Consequently, an insulin with 'intermediate' timing of action was sought to deal with postprandial hyperglycaemia. Surfen insulin, globin insulin and iso-insulin became available during 1938-1944 but were soon discontinued. In 1946, Neutral Protamine Hagedorn (NPH) insulin, a crystalline suspension, with protamine and insulin in 'isophane' proportions in the presence of zinc ions, with phenol and *m*-cresol as preservatives, emerged from Hagedorn's laboratory (42). NPH insulin reached the market in 1950 replacing the original two-vial system (protamine and buffer) as well as separate regular insulin and PZI injections. In the 1970s-80s, fixed ratio combinations of regular and NPH insulins (range 10:90 to 50:50) became available in an attempt to provide both meal and basal insulin requirements, administered once or commonly twice-daily.

The use of foreign proteins as retarding agents raised concerns, however Scott and Fisher demonstrated in 1935 that adding zinc ions prolonged the action of insulin (38). This effect was largely dependent on the physical state and size of the suspended zinc insulin particles (43). The subsequent Lente trilogy of insulins developed during in the 1950s, included a suspension consisting entirely of amorphous zinc insulin (semilente) with a shorter action than the microcrystalline insulin suspension (ultralente), and another with a 30:70 mixture (Lente insulin) that had with an intermediate duration, and was intended for once-daily administration (44).

Exploiting the solubility differences between porcine and bovine insulin at neutral pH also resulted in the development of a biphasic insulin, Rapitard (a mixture of 25% soluble porcine insulin and 75% bovine insulin crystals) in 1965 (45).

Following subcutaneous injection, NPH and Lente insulins generally have their peak action around 4-6 hours with a steady decline thereafter, with duration of action that varies according to the dose (46). As NPH and Lente insulin preparations are suspensions, that require thorough resuspension prior to injection, considerable variability occurred in the rate and amount absorbed and the subsequent glucose lowering effect (47,48). This remains a major and often ignored source of inaccuracy in their clinical use (49). Mixing regular and Lente insulin transforms the former into a semilente-like state due to the excess zinc in Lente insulin, the extent of which depends upon the proportions used and any delay in administration (50,51). This was a major contributor to the subsequent demise of Lente insulin. Bovine ultralente also generated neutralising antibodies that prolonged its action and obtunded regular insulin. Consequently, porcine and human ultralente became available in the early 1980's (52) with human ultralente insulin considered suitable as a once-daily basal insulin supplement (53).

3. Entering into and emerging out of a dark period in the history of insulin therapy (1930s to 1970s)

With the availability of insulin in 1922, expectations were understandably high given its transformational powers (54). Allen pronounced in 1930 that "every patient can now be expected to live out his natural life" (55). However, Joslin had cautioned in 1928 that "the disease is far from solved by insulin which marked the end of one era in diabetes management, not the end of diabetes" (56). Both were adamant that good glucose control, through a restricted carbohydrate diet, exercise, frequent testing and insulin adjustment,

could prevent complications. Unfortunately, this was not a view that was universally accepted at the time.

During the early years of the “insulin era” a dramatic reduction in mortality due to diabetic coma secondary to ketoacidosis was observed at the Joslin clinic in Boston, USA (57) (Figure 4). Instead, of being an acute, disgusting and fatal disease, diabetes soon became a chronic disease with accelerated, cardiorenal-vascular degenerative complications. From the late 1930s, as patients’

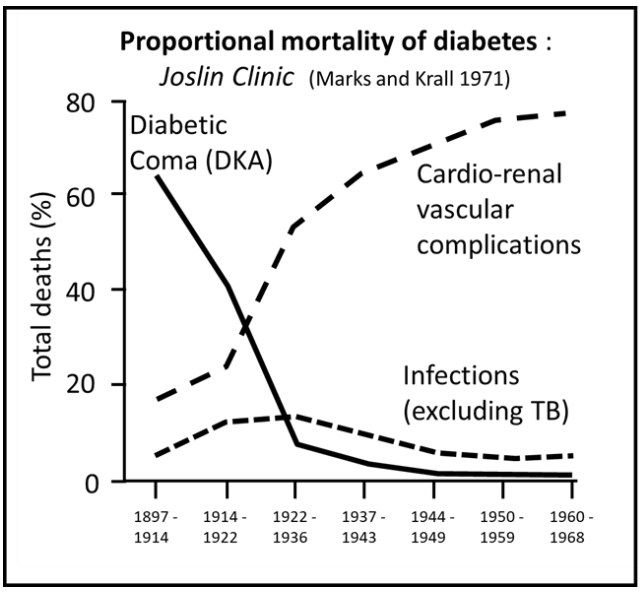

Figure 4

lives were extended, diabetes entered a dark period in its history.

With the initial availability of insulin, the mechanisms of its secretion and action were not known, and blood sugar measurements were infrequent. Glycosuria was the only index of control available to adjust insulin doses. It was not until the mid-1930s that diabetes was acknowledged as a disease “referable either to deficiency of insulin or to insensitivity to insulin” (58). Initially, multiple daily doses of short-acting regular insulin were used to control post-prandial hyperglycemia, but were not capable of satisfying nocturnal requirements.

Between 1936-1952, several protracted-acting insulins emerged (42, 43), and provided the convenience of once or twice- daily insulin dosing. However, all were suspensions, and with inadequate resuspension resulted in substantial day-to-day glycaemic variability (48). With few exceptions, these new ‘basal’ insulins were used alone with no regular insulin to cover mealtime requirements. The problem was compounded by the increasing adoption of free diets and the

practice of mixing regular insulin with these protracted-acting insulins. During this 20 year period debate raged about the aetiology of microvascular complications, the optimal method of treatment and the relevance of maintaining good metabolic control, targeting near normal blood sugar levels and preventing glycosuria. Edward Tolstoi, amongst others emphasised the negative effects of intensifying treatment on lifestyle with, in their view, no long-term benefit.

However, during this uncertain period in diabetic care, major scientific advances were being made.

The co-development of the radioimmunoassay (RIA) by Berson and Yalow in the 1950s (RIA) (Figure 3b), represents one of the major contributions to medical research in the last century (15). This occurred despite difficulties in the publication of their early work. Their developed immunoassay of endogenous plasma insulin in man in 1960 (59) revolutionised our understanding of diabetes and the physiology of glucose homeostasis. It also confirmed earlier findings from Himsworth in 1936 (58) that diabetes was either


Rosalyn Yalow

Figure 3b

predominantly an insulin deficient or resistant state. For the development of the radioimmunoassay technique, Yalow was awarded the Nobel Prize in Medicine or Physiology in 1977, some five years after the untimely death of Berson.

The 1960s saw Dorothy Crowfoot (later Hodgkin) (Figure 3c) reignite her former crystallographic work with insulin (60) to elucidated the 3- dimensional arrangement of the atoms in insulin using X-ray analysis of rhombohedral pig 2-Zn insulin crystals (61). She was supported by her team that included Margaret Adams, Eleanor and Guy Dodson, Tom Blundell, and Ted Baker. They deduced that the hexamer in 2-Zn insulin crystals was organised around a crystallographic three-fold axis with each hexamer consisting of three dimers with the monomers related by a two-fold axis perpendicular to and passing through the three-fold axis. For her pioneering work, Crowfoot was awarded the Nobel Prize for Chemistry in 1964.


Dorothy Hodgkin

Figure 3c

Several years later, Donald Steiner discovered proinsulin in 1967 that revolutionised our understanding of the molecular mechanisms of insulin biosynthesis in the pancreatic islets (62). Steiner and colleagues showed that proinsulin, a single polypeptide precursor (prohormone) could be cleaved to produce insulin, along with a connecting peptide segment (C-peptide) and a pair of basic amino acids (arginine and lysine).

Despite the magnitude of these ongoing scientific developments during the 1950-1960s, confusion remained in clinical circles about the optimal use of insulin. This was compounded by the initial results from the University Group Diabetes Program (UGDP) study in 1970 that reported a higher mortality in patients treated with insulin compared with patients receiving placebo (63). However, reanalysis of the study results years later, showed complete opposite findings (64). In 1976, the American Diabetes Association finally pronounced that the goals of therapy to prevent late complications should include

‘a serious attempt to achieve blood glucose levels as close to those in the non-diabetic state as feasible’.

In the late 1970s, several “pre-mixed” preparations with fixed ratios of regular and NPH insulin became available. The most common was a 30/70 regular/NPH ratio that was administered twice-daily using a “split-mixed” regimen. These premixtures which were widely adopted represented inappropriate modifications of the pharmacological profile of the constituent insulins (50). Resuspension, even under optimized experimental conditions, also failed to achieve good control. Their limited flexibility when titrated to target, could increase the risk for hypoglycemia and subsequent impaired awareness of hypoglycaemia was not uncommon (65). In real life patients often decrease their insulin dose to avoid hypoglycaemia, resulting in persistent hyperglycaemia and its associated long-term consequences.

Figure 5a (66) highlights the non-rational pharmacology (pharmacokinetics [PK]/pharmacodynamics [/PD]) of insulin after subcutaneous injection of a mixture of regular plus Lente insulin, revealing a deficiency in the early postprandial rise of insulin causing excess hyperglycemia, with an inappropriately residual high insulin level (absorption of the retarded insulin component) increasing the risk of hypoglycemia before lunch. Using separate injections for the meal (regular insulin), and a basal insulin supplement (NPH)

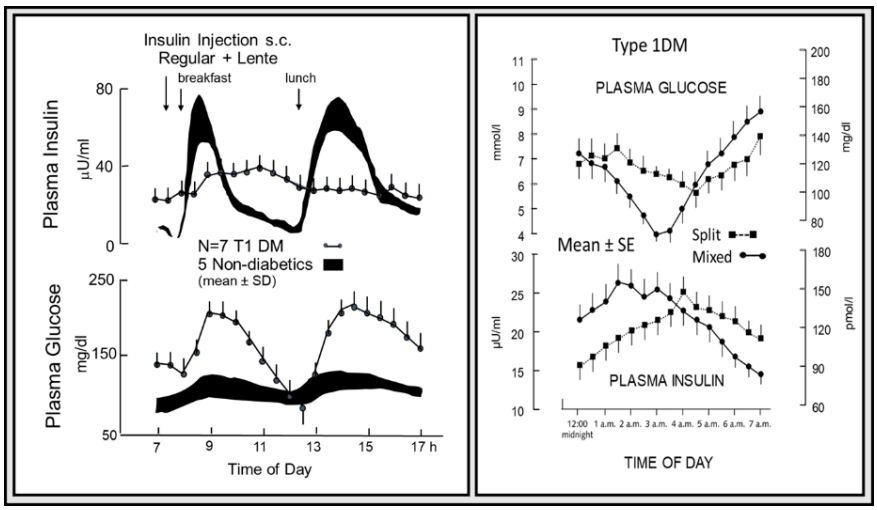

Figure 5a Figure 5b

improves post-meal blood glucose, and reduces the risk of nocturnal hypoglycaemia with improved fasting blood glucose the next morning, Figure 5b (67). Separate injections allow individuals to achieve a more meaningful titration of the two insulins.

Thankfully, the late 1970s saw the adoption of multiple daily insulin (MDI) regimens, combining basal and prandial insulins and, supported by self-monitoring of blood glucose (SMBG) levels. This made it possible to design and implement rational strategies of insulin replacement that more closely matched the normal physiological glucose response. In parallel, portable insulin delivery devices for continuous subcutaneous insulin infusion (CSII) became available. These changes facilitated the emergence from the earlier dark period in the history of insulin therapy. The original belief held by Joslin and others, that good glycaemic control was essential for the management of diabetes was eventually validated 30 years after his death, when in 1993, the landmark Diabetes Control and Complications Trial (DCCT) reported its key findings (68). The feasibility of achieving good control with physiological replacement of insulin via MDI or SCII in people with T1DM was demonstrated along with a reduction in microvascular and macrovascular complications that extended well beyond the trial period, thereby creating what we now refer to as the “legacy” effect of good

glycaemic control (69,70). The DCCT and the follow-up EDIC (Epidemiology of Diabetes Interventions and Complications) study, represent the basis of today's universal acceptance of the model of physiological insulin replacement.

In 1995, a relatively small study from Japan (Kumamoto) also observed that intensive insulin therapy prevented the onset, progression and severity of long-term microvascular disease in people with type 2 diabetes (71). These findings were soon overshadowed by the much larger United Kingdom Prospective Diabetes Study in T2DM (UKPDS) in 1998 that also demonstrated the short- and long-term benefits of intensive glucose control in people with type 2 diabetes (72, 73).

The DCCT/EDIC and UKPDS studies all provided irrefutable evidence supporting the need and benefits of improving glycaemic control, thereby encouraging further advancements in the development of new insulin preparations, improved means of delivery and monitoring in an attempt to safely achieve near normal glycaemia.

Table 1 summarises some of the key milestones in the evolution of insulin preparations, and the accompanying scientific achievements during the period 1922 to 1980.

Milestones influencing the development of insulin preparations (1922-1980)	
Insulin therapy	Scientific Developments
1921-22 Isolation and application of insulin	1926 Crystallisation of insulin
1923 Insulin standardisation	1928 Insulin is a protein
1936 Protamine insulinate, Protamine zinc insulin	1934 Zinc insulin crystallisation
1938-44 Surfen insulin, Globin insulin, Iso-insulin	1935 X-Ray photographs of insulin
1946 Isophane (NPH) insulin	1955 Primary structure of insulin
1951-52 Lente trilogy of insulins	1956 Radioimmunoassay of insulin
1959 Biphasic insulin (Rapitard)	1960 Human insulin sequence
1961 Neutral insulin solution	1967 Proinsulin structure
1970 Monocomponent insulin	1969 Tertiary structure of insulin
1980 Semi-synthetic human insulin available	1974 Chemical synthesis of human insulin
	1979 Biosynthesis of human insulin

Table 1

4. Era of Recombinant Insulin Preparations

4.1 Introduction of recombinant DNA (rDNA) technology

During the 1970's, Walter Gilbert and Frederick Sanger (Figure 3d) independently devised techniques for sequencing DNA and provided tools that enabled the manipulation of genes. For this work, they subsequently shared the 1980 Nobel Prize in Chemistry with Paul Berg. In 1977, Ullrich and colleagues cloned the rat insulin gene (74). These key advances in molecular biology and synthetic nucleotide chemistry, led to the synthesis of insulin, and several other mammalian polypeptide hormones by microbiological fermentation (75). Recombinant DNA (rDNA) technology provided a potentially limitless source of human insulin (76), and the opportunity for molecular modifications to enhance its pharmacological properties.

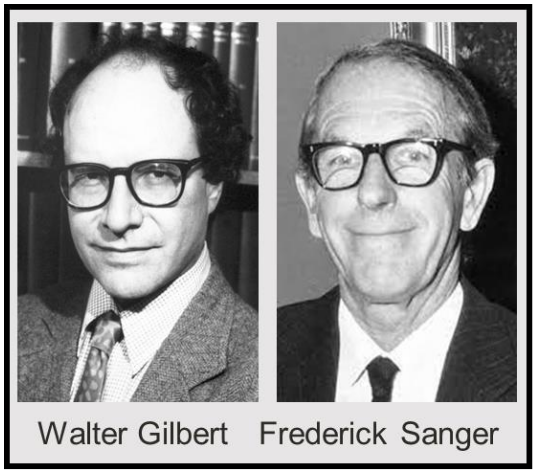

Figure 3d

In 1978, Genentech successfully synthesised human insulin A- and B-polypeptide chains by inserting DNA sequences encoding for the A and B chains into plasmids that were then cloned separately in a strain of *E.coli*. Following isolation and purification, the chains were converted to S-sulphonate salts to restore the disulphide bonds prior to being fused to form human insulin and undergo purification (77,78) (Figure 6 (79). Further development by Eli Lilly, resulted in the first-ever genetically engineered human insulin that became available for clinical evaluation in 1980 (80) and for clinical use in 1982.

A second method for the production of human insulin introduced in 1981 involved the use of DNA sequences encoding for human proinsulin, thereby minimising the purification process and increasing the yield (81). The steps leading to the proinsulin S-sulphonate were similar to those for the individual A- and B-chains. The derivative was then treated with a thiol reagent allowing the proinsulin molecule to fold and form the disulphide bonds before being purified and enzymatically converted, using a mixture of trypsin and carboxypeptidase B, to human insulin for final purification. Alternatively, in 1991, Novo Nordisk launched their recombinant human insulin production using yeast *(Saccharomyces cerevisiae)* to express a modified single-chain proinsulin like precursor molecule for conversion to human insulin (82). The different source of biosynthetic human insulins all possessed physical characteristics that were identical to the natural hormone with biological activities equivalent to those of highly purified porcine and semi-synthetic human insulins.

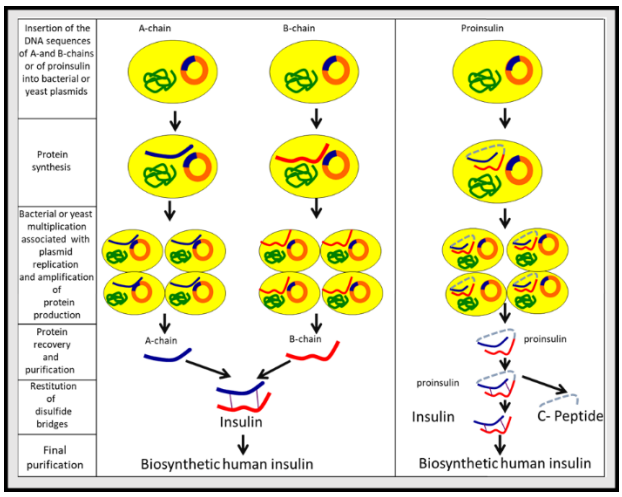

Figure 6

Recombinant DNA technology has also been pivotal in understanding the role of individual and groups of amino acids within the insulin molecule (Figure 7), indicating possible structural changes to improve storage stability, and modify its pharmacological properties.

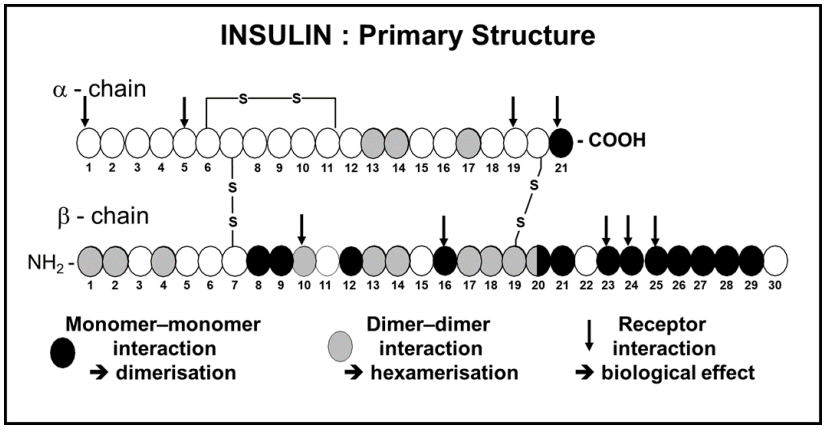

Figure 7

4.2 Fast-acting monomeric insulins

Postprandial hyperglycaemia is a contributory factor to inadequate glycaemic control and increased glucose variability, both of which are independent risk factors for cardiovascular complications (83,84). The existence of a lag phase in the absorption of regular insulin following subcutaneous administration was key to understanding the reasons for the inability to control postprandial hyperglycaemia (85). Equally important was the observation in the early 1970's that insulin associates into hexamers and dimers in concentrated neutral solutions in contrast to being in the monomeric state in the systemic circulation (86). This implied that insulins with a reduced propensity to self-associate would be more rapidly absorbed (87,88).

In the dimer, individual insulin molecules are held together predominantly by non-polar forces reinforced by four hydrogen bonds between B24 and B26 arranged as an anti-parallel B-sheet structure between the two carboxy-terminal strands of the B-chain. The amino acid residues involved in the association of two monomers into dimers are A21, B 8, 9,12,16,20,21 and 23-29.

The main strategies to counteract self-association include either (1) charge repulsion with already existing charge or introducing charge counterparts, (2) changing hydrophilic into hydrophobic interfaces, (3) replacement of metal binding sites or (4) by causing steric hindrance (Table 2).

Strategies for creating rapid-acting insulin analogues		
1. Charge repulsion: Half with existing charge Introducing charge counterparts	 Asp(B28) Asp (B9, Glu (B27) Lys (B29) Glu(B27),Asp(A21)	 Aspart insulin Glulisine insulin
2. Hydrophilicity into hydrophobic interfaces	Glu (B16),Glu (B27), Glu (B26)	
3. Removal od metal ions	Asp(B10)	
4. Steric hindrance: Reversal Deletion	 Lys (B28), Pro(B29) desB27	 Lispro/ Humalog Glulisine insulin

Table 2

The size of the insulin unit (association state) and the dissociation rate were found to be major determinants of the rate of absorption from the subcutaneous tissue. Monomeric insulins were absorbed 3-4 times faster than a non-dissociating hexameric insulin (87,88, 89). The potential clinical benefit of monomeric insulins became evident when given prior to a standard meal in people with T1 DM (90). Plasma profiles of monomeric insulin analogues compared with regular human insulin (RHI) when given immediately before a meal and were at least as effective as RHI when given 30 min earlier. The insulin analogue $B10^{Asp}$ was discontinued due to its prolonged affinity to the insulin receptor ("slow off rate") resulting in increased mitogenicity (91).

Various strategies were used to produce the currently available rapid-acting insulin analogues. Lispro insulin (Lys^{B28}-Pro^{B29}) approved in 1996 was based on the inversion of the two terminal amino acids of the B-chain (Figure 8). This was followed by insulin aspart (Asp^{B28}) in 2000 and glulisine (Lys^{B3}, Glu^{B29}) in 2004. Each of these rapid-acting prandial insulins improves postprandial glucose (PPG) control due to their faster rate of dissociation in the subcutaneous tissue from hexamers to dimers to monomers (lispro and aspart) or from dimers to monomers (glulisine). Their shorter action profile also lowers the risk of inter-prandial hypoglycaemia while allowing administration closer to meal time, thereby improving treatment satisfaction and quality of life (92).

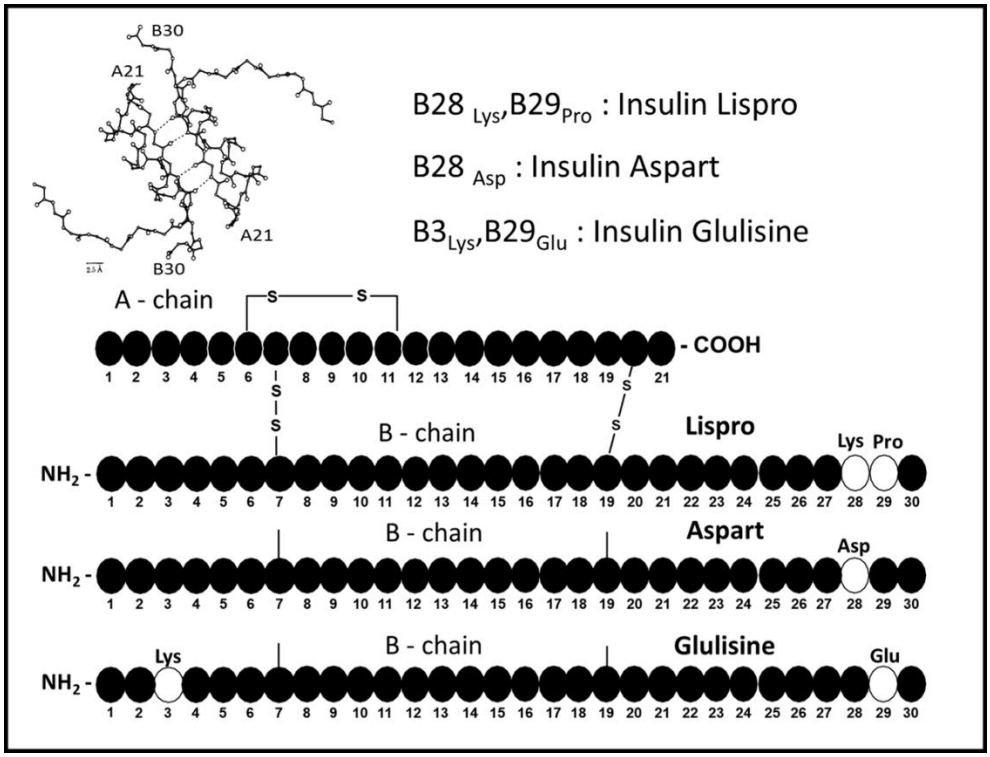

Figure 8

4.3 Ultra-Fast-acting insulin analogues

Recent evidence suggests that the 1h-PPG might be better at predicting diabetic complications compared with 2h-PPG (93). This recognition has stimulated developments aimed at producing faster-acting prandial insulins by improving insulin absorption, either via influencing the dissociation rate of hexamers to smaller units, by increasing local perfusion and/or by improving capillary permeability

at injection sites (94). This has resulted in reformulations of aspart (fast-acting aspart) and lispro (ultra-rapid lispro and BioChaperone Lispro).

Faster-acting aspart contains two additional excipients, niacinamide that increases local subcutaneous blood flow, and L-arginine that acts as a stabilizing agent. In contrast, ultra-rapid lispro contains citrate that enhances vascular permeability and treprostinil that increases local vasodilation at the injection site, without any measurable systemic exposure. Finally, BioChaperone Lispro contains citrate and BioChaperone BC222.30 which form a physical complex with insulin that protects it from enzymatic degradation while enhancing both its stability, solubility and rate of hexamer dissociation.

Compared with conventional aspart and lispro, faster aspart and ultra-rapid lispro given immediately before a test meal show reduced 1h-PPG excursion in people with T1DM and T2DM, although overall glycaemic control remains unchanged or marginally improved (95,96). Hypoglycaemia risk is essentially unchanged and sometimes increased, which may be due to the type of accompanying basal insulin used, suggesting the need to optimize basal insulin supplementation (94). Despite these positive advancements, delivery via the subcutaneous route may never adequately accommodate the nutritional vagrancies of normal life. Even the fastest acting insulin analogues still require when possible, a gap between injecting and eating to minimise post-prandial glucose rise.

When administered by CSII, faster aspart and ultra-rapid lispro significantly improve 1h-PPG compared to insulin aspart and lispro, respectively (97,98). Despite initial concerns related to compatibility in CSII devices (94), faster aspart and ultra-rapid lispro have now been approved for clinical use. BioChaperone Lispro remains in Phase 3 trials.

4.4. Long-acting 'basal' rDNA insulin analogues:

Once Daily to Once weekly insulin analogues

The inherent limitations of the surrogate basal insulin suspensions NPH and Lente insulin (99) have stimulated ongoing development of peak less prolonged-acting soluble insulins for over 50 years. Successful strategies used over the last 25 years to achieve these insulin profiles have involved on achieving (1) low insulin solubility at physiological pH by increasing the isoelectric point of insulin, or (2) conjugating insulin to a fatty-acid chain of variable lengths.

4.4.1 Di-arginyl insulin and insulin glargine (U100)

Early attempts involved substitutions at the C-terminal end of the B-chain by addition of positive charges that shifted the isoelectric point towards a neutral pH. One early preparation in 1989 was OPID 174 (NovoSol Basal), that had a slow and consistent rate of dispersal from the subcutaneous tissue, compared with human ultralente insulin (100). However, the low bioavailability and variable action profile resulted in this insulin being discontinued.

Although retaining the di-arginyl ($Arg^{B31}Arg^{B32}$) amino acids at the C-terminus of the B-chain was then considered a logical next step, since it possessed only 50% activity of native insulin, reliance only on a shift in the iso-electric point was deemed insufficient (101). Arginine residues at the C-terminus of the B-chain are also important for inter-hexamer interactions and crystal stability against dissolution, due to a higher packing density of the crystals. Substitution of asparagine by glycine at A21 allowed binding of a seventh phenol at the periphery of the insulin hexamer to complement, the six at the centre which stabilise the dimer-dimer interactions. This combination resulted in the development of $A21^{Gly}$,$B31^{Arg}$,$B32^{Arg}$-insulin (glargine) with 30ug/ml of zinc. Insulin

glargine had a much slower rate of subcutaneous absorption (102) and a longer duration of action than NPH insulin, and was suitable for once daily administration (103). As it was a soluble formulation, re-suspension prior to injection was not required but it was immiscible with RHI.

While being soluble in the acidic pH of the insulin vial, glargine at neutral pH of the subcutaneous tissue is quickly transformed into an amorphous crystalline precipitate before dissolution into hexamers and dissociating into dimers and monomers. This accounts for its delayed absorption and extended duration of action (Figure 9).

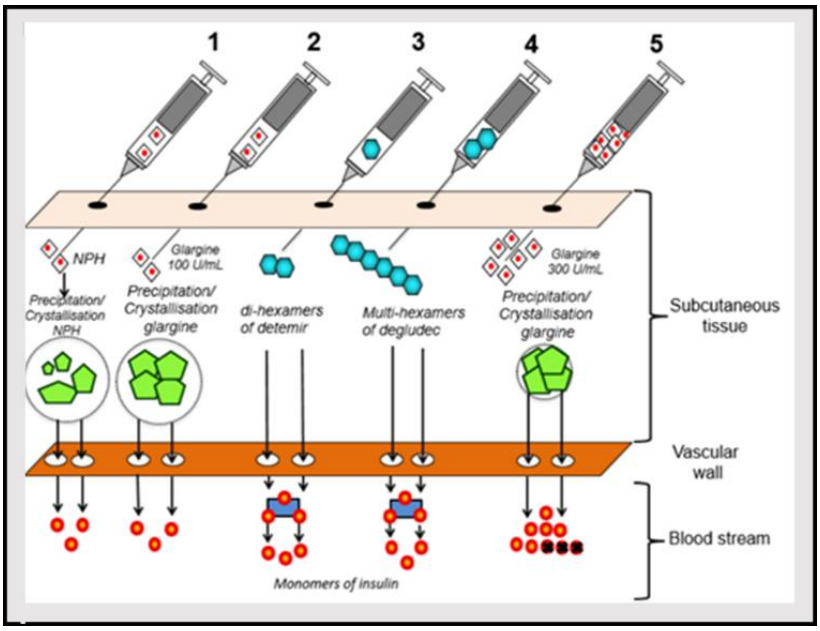

Figure 9

Subcutaneously administered glargine behaves like a 'prodrug,' and is quickly metabolised into its active components, primarily M1 (des B31,32 -HI) and M2 (des-B30Thr-HI) (104), thereby eliminating any mitogenic potential known to be associated with the parent compound (105).

The seminal study by Lepore and colleagues published in 2000, showed the pharmacokinetic and pharmacodynamic differences between glargine compared with NPH, ultralente, and CSII (106). Compared with NPH, glargine showed a relatively consistent peak-less concentration-time profile that translated to a lower risk of hypoglycaemia despite, similar glycaemic control (107). The Treat-to-

Target trial in people with T2DM, showed that while approximately 60%

achieved HbA1c ≤7.0% with NPH and glargine, 25% more reached this target without hypoglycaemia on glargine (108). Initial concerns about the risk of cancer and cardiovascular safety with glargine were allayed in the long-term ORIGIN (Outcome Reduction with Initial Glargine Intervention) study (109).

4.4.2 Acylated insulins:

An alternative method to prolong the action of insulin was pursued by NovoNordisk in the late 1990s. It involved fatty acid acylation (lipidation) of insulin, based on the principle of reversible binding of fatty acids to serum albumin. Consequently, human insulin analogues were modified by linking a fatty acid moiety to the epsilon-amino group of B29Lys of desB30 human insulin (79) (Figure 10).

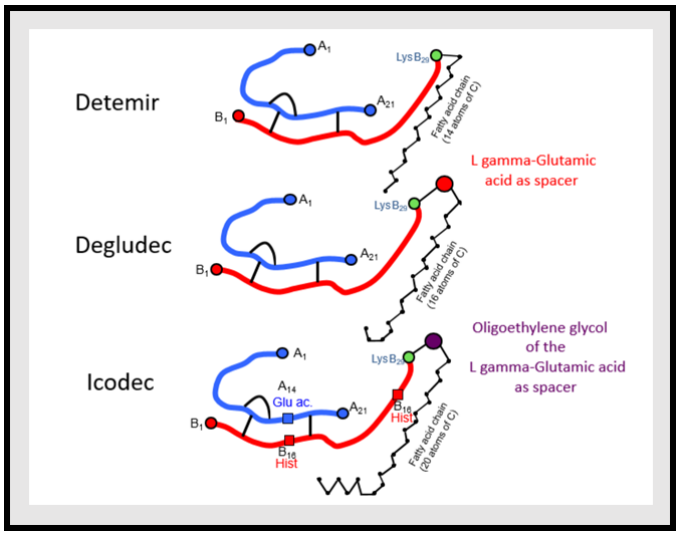

Figure 10

4.4.2.1. Insulin detemir

Insulin detemir (B29Lys-tetradecanoyl-des B30 human insulin) was the first of these neutral soluble acylated generation of basal insulin preparations, having a C14 fatty acid chain (myristoyl fatty acid) bound to the epsilon-amino acid group of $B29^{Lys}$ of desB30 human insulin (Figure 10).

Its protracted and consistent action was due to self-association to di-hexamers in the subcutaneous tissue and reversable binding of the high affinity fatty acid chain to albumin in the subcutaneous and

intravascular compartments (110). The lower affinity for the insulin receptor meant a 4-fold increase in the active component was needed for similar potency to human insulin (111). Available since 1996, detemir has an overall glucose lowering effect equivalent to NPH but with lower within-subject variability, lower risk of hypoglycaemia and reduced weight gain (112). When administered once-daily at the same dose, detemir was shown to be inferior to 100 U/ml (U100) in glucose lowering and duration of action among people with T1DM (113) and T2DM (114); -primarily in the obese (115). This has resulted in detemir being administered twice-daily in about half the people with T2DM patients (116).

4.4.2.2 Insulin degludec

In a further development, the fatty acid side chain was extended with a diacidic hexadecandioyl (C16) fatty acid to produce insulin degludec. It has a low affinity for hIGF-1 receptors that is comparable to human insulin (117). The C16 fatty acid chain is attached at B29Lys via a Gamma-glutamic acid spacer. While existing as stable di-hexamers in the pharmaceutical formulation, it undergoes multi-hexamer assembly in the subcutaneous tissue due to the slow diffusion of phenol (118). The subsequent extrusion of zinc results in dissociation of the insulin hexamers into dimers and monomers that enter the systemic circulation where it reversibly binds to serum albumin. Pharmacological studies show degludec has a flat and stable glucose lowering effect with a half-life of approximately 25 hours (119), based on the total, (not the free/active) degludec concentrationestimated on the total and not the free/active degludec concentration. In clinical studies degludec shows equivalent glycaemic control to glargine U100 but with a significantly lower risk of nocturnal hypoglycaemia in people with T2DM (120) and T1DM (121). Some initial concerns about cardiovascular safety were allayed in the large double-blind DEVOTE study, that confirmed

the cardiovascular safety of degludec was equivalent to that of glargine (122). The structural characteristics of insulin degludec also allowed development of co-formulations with rapid-acting insulin aspart and the glucagon-like peptide 1 analogue liraglutide (123,124).

4.4.1.2 Glargine U300

The original glargine U100 has been reformulated at a higher concentration (300 U/ml or U300). In the subcutaneous tissue glargine U300 has a depot surface area that is half that of glargine U100, thereby retarding its dissolution rate by half that, results in a more consistent and prolonged action (125). Its metabolism is identical to U100, reaffirming that GlyA21-human insulin is the active component. Glargine U300 shows equivalent glycaemic control to glargine U100 in people with T2DM, although a slightly higher dose (~15%) is required possibly due to local enzymatic degradation at the site of injection (126). Also, a lower risk of hypoglycaemia (day and night), especially during the initial titration period is observed with glargine U300 (127). In people with T1DM, when administered at equipotent doses glargine 300 shows a smoother and more prolonged absorption than glargine U100. This results in a lower risk of nocturnal hypoglycaemia and better afternoon plasma glucose (128).

The Head-to-Head comparative trial (Bright study) between glargine U300 and insulin degludec in insulin naïve T2DM, showed equivalent glycaemic control and weight changes over 24 weeks (129). The only slight difference was seen during the titration period with a less hypoglycaemia with glargine. In contrast, the CONCLUDE study that compared degludec U200 with glargine U300, no significant difference in the of overall symptomatic hypoglycaemia was (130). It

is apparent that these contrasting findings largely reflect differences in study design, execution and study populations.

These new-generation basal insulins show equivalent glycaemic control, a lower risk of hypoglycaemia, and reduced glycaemic variability than their earlier counterparts helping to erase some of the barriers to insulin initiation and intensification.

4.4.2.3 Insulin icodec

A recent addition to the acylated generation of basal insulins, insulin icodec, has emerged over the last year. This compound includes a C20 icosane fatty diacid side chain that is linked to desB30 HI with three amino acid modifications at A14, B16 and B25 that help to maintain molecular stability, solubility, and reduce enzymatic degradation and receptor mediated clearance (131). The estimated half-life of approximately 200 hours, making it suitable for once-weekly dosing. In insulin naïve people with T2DM, overall glycaemic control (HbA1c) with icodec is similar to glargine U100, although slightly more participants achieved HbA1c targets with a longer time in range (132). Although infrequent, hypoglycaemic events rates were higher with icodec despite a higher dosage requirement with glargine. These preliminary findings are encouraging, however, the lower flexibility of icodec may limit safety during titration. Other molecules based on Fc recycling and lymphatic absorption are in development.

Table 3 summarises the major milestones in the development of recombinant DNA (rDNA) derived rapid, ultra-rapid and prolonged-acting insulin analogues during the period 1980-2021. Related scientific advance in the use of recombinant DNA technology during the period 1977-2019 are highlighted.

Major Milestones in rDNA Insulin analogues (1980-2021)	Prolonged-acting insulin analogues	M
1988 **Monomeric Insulin analogues**	**Isoelectric point shift towards neutral pH:**	1977
Rapid-acting Insulin analogues	1985 Arg ^{B31}, Arg^{B32}-Insulin; di-arginyl insulin	
1996 LysPro Lispro insulin (USA, EU)	1988 OPID-174	1979
1999 Insulin Aspart (EU)	2000 Gly^{A21}, Arg ^{B31}, Arg^{B32} -I: glargine U100	1980
2004 Insulin Glulisine (USA, EU)	2013 Gly^{A21}, Arg ^{B31}, Arg^{B32} -I: glargine U300	1981
Ultra-Rapid·acting Insulin analogues	**Acylation of insulin with fatty acids:**	
2017 Faster Insulin Aspart (FIAsp)	2004 Lys^{B29}-tetradecanoyl-des^{B30}-I; detemir	1986
2020 Ultra-Rapid Lispro Insulin (URLI)	2015 Lys^{B29}ɣ-glutamyl-hexadecanediol-des^{B30}-I: insulin degludec	2013
NA BioChaperone Lispro Insulin	NA Lys^{B29}ɣ-glutamyl-hexadecanediol-des^{B30}-I: insulin icodec	2019
Approval dates; NA = Not approved	Approval dates; NA = Not approved	

Table 3

5.Concluding remarks

The epoch-making discovery of insulin by the Toronto team, led by Banting and Macleod, with vital input from Collip and the technical support of Best, heralded the birth of insulin therapy in 1922. The application of insulin since then has transformed the lives of countless people with diabetes across the globe. Major milestones in the first 60 years included the purification, standardisation, prolongation of action conversion from animal to human insulin. The advent of recombinant technology 40 years ago has revolutionised the production of insulin, allowed modifications (‘designer insulins’) and co-formulations to be produced in an attempt to replicate the normal basal and nutrient related insulin secretory patterns. Despite the impressive achievements by the pharmaceutical industry, the demand on insulin requiring patient remains relentless, as they try to navigate their way between hyper- and hypoglycaemia each day and night to prevent unwanted complications.

The expectation during the next centenary is that diabetes in all its forms will be cured obviating the need for insulin, when the ‘Flame

of Hope' outside Banting's former residence can finally be extinguished, signalling the event.

In the meantime, efforts to further modify and re-formulate insulin will continue, while also exploring different routes and modes of delivery aided by technical advancements in glucose monitoring. Finally, in today's world access to this life-giving treatment, although not a panacea, remains woefully inadequate, especially, although not exclusively, in low- and middle-income countries (133). Ensuring insulin and future innovations in insulin therapy become available to those in need, remains a global priority for the next centenary.

Word count 6357

Title: Insulin Centennial: Milestones influencing the development of insulin preparations since 1922.

Acknowledgments: Authors would like to express their special thanks to the Library staff at the Swansea Bay University Health Board, Moriston Hospital, Swansea, Wales, UK. Also, thanks to Dr Melanie Moore, Senior Scientist at the National Institute Biological Standards and Controls, Potters Bar, UK, for data on international standards for insulin over the years and to Dr Dan Quillan for constructive discussions and suggestions during the preparation of the review.

Conflict-of- interest statements:

Owens DR has received honoraria for lectures and advisory board activities from Boehringer Ingelheim, Novo Nordisk, Roche Diagnostics and Sanofi.

Monnier L has no conflict of interest relating to this article

Ceriello A has participated in advisory boards, provided consultancy and lectures to Astra Zenica, Berlin Chemie, Eli Lilly, Novo Nordisk and Roche Diagnostics.

Bolli GB has received research grant support and honoraria for advising and consultation from Sanofi and Menarini.

References

1.Allen FM. The Present outlook of diabetic treatment. Trans Assoc Am Physicians 1917; 32: 138-148

2.Banting FG. Diabetes and Insulin, Nobel Laureate Lecture, Stockholm 15th September 1925, Can Med Assoc J 1926;16:221-232

3.Bliss M. The Discovery of Insulin. McClelland and Stewart Limited, The Canadian Publishers Toronto, Ontario, Canada 1982

4.Bliss M. Banting A Biography. McClelland and Stewart Limited, The Canadian Publishers, Toronto, Ontario, Canada 1984

5.Joslin EP. The unkown diabetic. Postgrad Med 1948;4: 302-306

6. Bliss M. JJR Macleod and the discovery of insulin (Special lecture). Quarterly J of Exp Physiol 1989; 74:87

7. Bliss M. The eclipse and rehabilitation of JJR Macleod, Scotland's insulin laureate. J R Coll Physicians Edinb 2013; 43:366–73

8. Roth J, Quereshi S, Whitford I, Vranic M, Khan CR, Fantus IG, Dirks JH. Insulin's discovery: New insights on its ninetieth birthday. Diabetes Metab Res Rev 2012; 28: 293–304

9.Joslin EP. The routine treatment of diabetes with insulin. Trans Assoc Am Physicians 1923; 38:374-81

10.Banting FG, Best CH, Collip JB, Campbell WR, Fletcher AA. Preliminary Report Pancreatic Extracts in the Treatment of Diabetes Mellitus. Can Med Assoc J 1922;12:141-146

11.Colwell AR. Fifty years of Diabetes in Perspective. Diabetes 1968; 17:599-610

12.Banting FG, Campbell WR, Fletcher AA. Further Clinical Experience with Insulin (Pancreatic Extracts). Brit Med J 1923;i:8-12

13. Brange J. Galenics of Insulin: The physico-chemical Aspects of Insulin and Insulin Preparations. Springer-Verlag 1987: p1-14

14.Lawrence RD. Local insulin reactions. Lancet 1925.i:1125-1126

15.Abel JJ. Crystalline insulin. Proc Natl Acad Sci (Wash) 1926;12:132-136.

16.Scott DA. Crystalline insulin. Biochem J 1934;28:1592-1602

17.Jorpes JE. Recystallised insulin for diabetic patients with insulin allergy. Arch Intern Med 1949;83:363-371

18.Berson SA, Yalow RS Bauman A, Rothschild MA, Newerly K. Insulin-I^{131} metabolism in human subject: demonstration of insulin binding globulin in the circulation of insulin-treated subjects. J Clin Invest 1956; 170-190

19.Schlichtkrull J, Brange J, Christiansen AaH et al. Monocomponent insulin and its clinical implications. Horm Metab Res (Suppl Ser) 1974;5:134-143

20.Bloom SR, Barnes AJ, Adrian TE, Polak JM. Autoimmunity in diabetics induced by hormonal contaminants in insulin. Lancet 1979;i:14-17

21.Jorgensen KH, Hallund O, Heding LG et al. Estimation of insulin purity in light of developments in analytical methods. In: Guerigian JL, Bransome E D, Outschoorn A S (eds) Hormone Drugs, United States Pharmacopeial Convention, Rockville, Maryland 1982: p139-147

22.Sanger F, Thompson EOP. The amino-acid sequence in the glycyl chain of insulin.1. The identification of lower peptides from partial hydrolysates. Biochem J 1953;53(3):353-366

23.Nicol DSHW, Smith LF. Amino-acid sequence of Human Insulin. Nature 1960;187:483-485

24.Sieber P, Kamber B, Hartmann A, Johl A, Riniker B, Rittel W. Totalsynthese von Humaninsulin unter gezielter Bildung der Disulfid-bindungen. Helv Chim Acta 1974;57:2617-2621

25.Obermeier R, Geiger R. A new semi-synthesis of human insulin. Hoppe-Seyler's Z Physiol Chem 1976;357:759-767

26.Homandberg GA, Mattis J A, Laskowski M. Synthesis of peptide bond by proteinases. Addition of organic cosolvents shifts peptide bond equilibria towards synthesis. Biochem 1978;17:5220-5227

27.Morihara K, Oka T, Tsuzuki H. Semi-synthesis of human insulin by trypsin-catalysed by replacement of Ala-B30 by threonine in porcine insulin. Nature 1979:280:412-413

28.Markussen J. Human insulin by tryptic transpeptidations of porcine insulin and biosynthetic precursors. MTP Press Ltd: England; 1987,

29.Heding FG, Larsson Y, Ludvigsson J. The immunogenicity of insulin preparations. Antibody levels before and after transfer to highly purified porcine insulin. Diabetologia 1980;19:511-515

30.Heding LG, Marshall MO, Persson B et al. Immunogenicity of monocomponent human and porcine insulin in newly diagnosed type 1 (insulin-dependent) diabetic children. Diabetologia 1984; 27(Suppl) :96 –98

31.Banting FG, Best CH, Collip JB, MacLeod JJR, Noble EC. The effect of pancreatic extract (insulin) on normal rabbits. Amer J Physiol 1922; 62:162-176

32.Sahyun M, Blatherwick NR. “The rabbit method of standardizing insulin.” Amer J of Physiology-Legacy Content 76.3 1926: 677-684

33.Heinemann L, Beals JM, Malon J, Anderson J, Jacobsen JG, Sinha V. Concentrated insulins: History and critical reappraisal. J of Diabetes 2019;11:292-300

34.Lacey AH. The Unit of Insulin. Diabetes 1967:16:198-200

35.Sinding C, Making the Unit of Insulin: Standards, Clinical Work, and Industry, 1920-1925. Bull Hist Med 2002; 76:231-270

36.Best CH. The prolongation of insulin action. Symposium on hormones (Sigma XI Lect) 1937:362-377

37.Hagedorn HC, Jensen BN, Krarup NB, Woodstrup I. Protamine insulate. JAMA 1936;106:177-180

38.Scott DA, Fisher AM. The effect of zinc salts on the action of insulin. J Pharmacol Exp Ther 1935;55:206-221

39. Lawrence RD, Archer N. Some experiments with protamine insulate.

40. Scott DA, Fisher AM. Studies on insulin with protamine. J Pharmacol Exp Ther 1936;58:78-92

41. Himsworth HP. Protamine insulin and zinc protamine insulin in the treatment of diabetes mellitus. Brit Med J 1937; 1:541-546

42.Krayenbuhl C, Rosenberg T. Crystalline protamine insulin. Rep. Steno Hosp (Copenhagen) 1946;1:60-73

43.Hallas-Moller K, Petersen K, Schlichtkrull J. Crystalline and amorphous insulin-zinc compounds with prolonged action. Science 1952;116:394-398

44.Stowers JM, Nabarro JDN. Clinical experience of the insulin zinc suspensions. Brit Med J 1955;i:68-71

45.Schlichtkrull J, Munck O, Jersild M. Insulin Rapitard and Insulin Actrapid. Acta Med Scand 1965;177:103-113

46.Owens DR, Jones IR, Birtwell AJ. Study of porcine and human isophane (NPH) insulin in normal subjects. Diabetologia 1984; 26:261-265

47.Lauritzen T, Faber O K, Binder C. Variation in 125I-insulin absorption and blood glucose concentration. Diabetologia 1979;17:291-295

48.Lucidi P, Porcellati F, Marinelli A et al. Pharmacokinetics and Pharmacodynamics of NPH insulin in type 1 diabetes: The importance of appropriate resuspension before subcutaneous injection. Diabetes Care 2015; 38:2204-2210

49.Jehle PM, Micheler C, Jehle DR, Breiting D, Boehm BO. Inadequate suspension of neutral protamine Hagedorn (NPH) insulin in pens. Lancet 1999;354:1604-1607

50.Nolte MS, Poon V, Grodsky GM, Forsham PH, Karam JH. Reduced solubility of short-acting soluble insulins when mixed with longer-acting insulins. Diabetes 1983;32:1177-1181

51.Owens DR, Vora JP, Jones IR. Soluble and lente human insulin mixtures in normal man. Diabetes Care 1988;7:35-40

52.Owens DR, Vora JP, Heding LG et al. Human, porcine and bovine ultralente insulin: subcutaneous administration in normal man. Diabetic Med 1986; 3:326-329

53.Holman RR, Steemson J, Darling P, Reeves WG, Turner RC. Human ultralente insulin. Br Med J 1984;288:665-668

54.Geyelin HR, Harrop G, Murray MF, Corwin E. The use of Insulin in Juvenile Diabetes. J Metab Res 1922;2:765-791

55.Allen FM. Methods and results of diabetic treatment. New Engl J Med 1930;203:1133-1139

56.Joslin EP. Treatment of Diabetes Mellitus.4th ed. Philadelphia, Lea & Febinger, 1928

57.Marks HH, Krall LP. Onset, Course, Prognosis and Mortality in Diabetes Mellitus, in Joslin's Diabetes Mellitus 11th Edition, Eds Marble A, White P, Bradley R F, Krall L, Lea and Febinger , Philadelphia 1971: p209-254

58.Himsworth HP. Diabetes Mellitus Its Differentiation into Insulin-Sensitive and Insulin-Insensitive Types. Lancet 1936; i:127-130

59.Yalow RS, Berson SA. Immunoassay of endogenous plasma insulin in man. J Clin Invest 1960;39:1157-1175

60.Crowfoot DM. X-Ray Single Crystal Photographs of Insulin. Nature 1935;135:591-592.

61.Adams MJ, Blundell TL, Dodson EJ et al. Structure of Rhombohedral 2 Zinc Insulin Crustals. Nature 1969;224:491-495

62.Steiner DF Proinsulin and the biosynthesis of insulin. N Engl J Med 1969;280:1106-1113

63.University Group Diabetes Programme (UGDP) Study. Diabetes 1970;19 (Suppl 2):785-830

64.Genuth S. Exogenous Insulin administration and cardiovascular risk in Non-insulin-dependent and Insulin-dependent diabetes mellitus. Ann Intern Med 1996;124:104-109

65.Fanelli CG, Paramore DS, Hershey T et al. Impact of nocturnal hypoglycaemia on hypoglycemic cognitive dysfunction in type 1 diabetes. Diabetes 1998;47(12):1920-1927

66.Dimitriadis G, Gerich J Effect of twice daily subcutaneous administration of a long-acting somatostatin analog on 24;hour plasma glucose profiles in patients with insulin-dependent diabetes mellitus. Horm Metabol Res 1985;17:510-511

67.Fanelli CG, Pampanelli S, Porcellati F et al. Administration of neutral protamine Hagedorn insulin at bedtime versus with dinner in type 1 diabetes mellitus to avoid nocturnal hypoglycaemia and improve control. A randomized, controlled trial. Ann Int Med 2002;136:504-514

68.The Diabetes Control and Complications Trial Research Group, The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. N Engl J Med 1993;329:977–986

69.The Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications Research Group, Retinopathy and Nephropathy in Patients with Type 1 Diabetes Four Years after a Trial of Intensive Therapy. *N Engl J Med* 2000;342: 381-9

70.The Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications Study at 30 years: Nathan DM, Bayless M, Clearly P, Genuth S, Gubitosi-Klug R, Lachin JM, Lorenzi G, Zinman B for the DCCT/EDIC Research Group. Diabetes 2013;62(12):3976-3986

71.Ohkubo Y, Kishikawa H, Araki E et al. Intensive therapy prevents the progression of diabetic microvascular complications in Japanese patients with non-insulin dependent diabetes mellitus: a randomised prospective 6-year study. Diabetes Res Clin Pract 1995;28:103-117

72.Intensive blood-glucose control with sulphonylureas or insulin compared with conventional treatment and risk of complications in

patients with type 2 diabetes (UKPDS 33). UK Prospective Diabetes Study (UKPDS) Group. Lancet. 1998;352:837-53.

73.Holman RR, Paul SK, Bethel MA, Mathews DR, Neil HA. 10-Year Follow-up of Intensive Glucose Control in Type 2 Diabetes. *N Engl J Med*, 1998;359:1577-89

74.Ullrich A, Shine J, Chirgwin J et al. Rat insulin genes: construction of plasmids containing the coding sequences. Science 1977;196:1313-1319

75.Gilbert W, Villa-Komaroff L. Useful proteins from recombinant bacteria. Sci Am 1980;242:74-94

76.Miller WL, Baxter JD. Recombinant DNA - A new source of Insulin Diabetologia 1980;18:431-436

77.Goeddel DV, Kleid DG, Bolivar F et al. Expression in *Escherichia Coli* of chemically synthesised genes for human insulin. Proc Natl. Acad. Sci. USA 1979;76: 106-110

78.Chance RE, Kroeff EP, Hoffman JA, Frank BH. Chemical, physical and biological properties of biosynthetic human insulin. Diabetes Care 1981b;4:147-154

79.Monnier L, Colette C, Owens D. Si l'ere des insulins semi-synthetiques et biosynthetiques nous etait contee. Med Mal Metab 2021;15:3533-3552

80.Keen H, Glynn A, Pickup J C et al. Human insulin produced by recombinant DNA technology – Safety and hypoglycaemic potency in healthy men. Lancet 1980;ii:358-401

81.Frank BH, Pettee JM, Zimmermann RE, Burck PJ, The production of human proinsulin and its transformation to human insulin and C-peptide. In: Rich O H, Gross E (eds), Peptides: Synthesis-Structure-Function. Proceedings of the Seventh American Peptide Symposium, Rockford II, Pierce Chemical Co 1981:729-783

82.Markussen J, Damgaard U, Diers I et al. Biosynthesis of human insulin in yeast via single-chain precursors. In:D. Theodoropoulos (ed) Peptide 1986, de Gruyter, Berlin, 1986:189-194.

83.Ceriello A. Point: postprandial glucose levels are a clinically important treatment target. Diabetes Care 2010;33:1905-1907.

84.Ceriello A, Monnier L, Owens D. Glycaemic variability in diabetes: clinical and therapeutic implications. Lancet Diabetes Endocrinol 2019;7:221–230.

85.Binder C. Absorption of injected insulin. Acta Pharmacol Toxicol 1969;Suppl 2:1-87

86.Blundell T, Dodson G, Hodgkin D, Mercola D. Insulin: the structure in the crystal and its reflection in chemistry and biology, Adv Protein Chem 1972;26:279-402

87.Brange J, Owens DR, Kang S, Volund A. Monomeric insulins and their experimental and clinical implications. Diabetes Care 1990;13(9): 923-954

88.Brange J, Volund A. Insulin analogues with improved pharmacokinetic profiles. Adv Drug Delivery Rev 1999;35:307-335.

89.Vora JP, Owens DR, Dolben J et al. Recombinant DNA derived monomeric insulin analogue: comparison with soluble human insulin in normal subjects. Br Med J 1988;297:1236-39

90.Kang S, Creagh FM, Peters JR et al. Comparison of subcutaneous human insulin and Insulin analogues (Asp^{B9}, Glu^{B27}; Asp^{B10}; Asp^{B28}) on meal-related plasma glucose excursions in type 1 diabetic subjects. Diabetes Care 1991;14: 571-577

91.Hansen BF, Kurtzhals P, Jensen AB, Dejgaard A, Russell-Jones D. Insulin X10 revisited: a super mitogenic insulin analogue. Diabetologia 2011;54:2226-2231

92.Home P D. The pharmacokinetics and pharmacodynamics of rapid acting insulin analogues and their clinical consequences. Diabetes Obes Metab. 2012;14:780-788.

93.Ceriello A. Targeting one-hour postmeal glucose: is it time for a paradigm switch in diabetes management? Diabetes Technol Ther 2017;19:493-97.

94.Owens DR, Bolli GB. The continuing quest for better subcutaneously administered prandial insulins: a review of recent developments and potential clinical implications. Diabetes Obesity and Metabolism 2020;22:43-754

95.Haahr H, Heise T. Fast-Acting Insulin Aspart: a review of its pharmacokinetic and pharmacodynamic properties and the clinical consequences. Clin Pharmacokinet 2020; 59:155-172.

96.Klaff L, Cao D, Dellva MA et al. Ultra-rapid lispro improves postprandial glucose control compared with lispro in patients with type 1 diabetes: results from the 26-week PRONTO-T1D Study. Diabetes Obes Metab 2020;22:1799-1807

97.Evans M, Ceriello A, Danne T et al. Use of fast-acting insulin aspart in insulin pump therapy in clinical practice. Diabetes Obes Metab. 2019;21:2039-2047.

98.Warren M, Bode B, Cho JI et al. Improved postprandial glucose control with ultra- rapid lispro versus lispro with continuous subcutaneous insulin infusion in type 1 diabetes: PRONTO-Pump-2. Diabetes Obes Metab 2021 Mar 9. doi: 10.1111/dom.14368.

99.Owens DR, Zinman B, Bolli GB. Insulin today and beyond. Lancet 2001;358:739-746

100.Jorgensen S, Vaag A, Langkaer L, Hougaard P, Markussen J. NovoSol basal: pharmacokinetics of a novel soluble long acting insulin analogue. BMJ 1989;299:415-419

101. Hilgenfeld R, Seipke G, Berchtold H, Owens DR, The Evolution of Insulin Glargine and its continuing contribution to Diabetes Care. Drugs 2014;74:911-927

102. Owens DR, Coates PA, Luzio SL, Tinbergen J P, Kurzhalls R, Pharmacokinetics of ^{125}I-labelled insulin glargine (HOE 901) in Healthy men: Comparison with NPH insulin and the influence of different subcutaneous injection sites. Diabetes Care 2000;23:813-819

103. Heinemann L, Linkeschova R, Rave K, Hompesch B, Sedlak M, Heise T. Time-action profile of the long-acting insulin analog insulin glargine (HOE901) in comparison with those of NPH insulin and placebo. Diabetes Care 2000;23(5):644–649.

104.Kuerzel GU, Shukla U, Scholtz HE et al. Biotransformation of insulin glargine after subcutaneous injection in healthy subjects. Curr Med Res Opin2003;19:34-40

105.Kurtzhals P, Chaffer L, Sorensen A et al. Correlation of receptor binding and metabolic potencies of insulin analogs designed for clinical use. Diabetes 2000;49:999-1005

106.Lepore M, Pampanelli S, Fanelli C et al. Pharmacokinetics and pharmacodynamics of subcutaneous injection of long-acting insulin analog glargine, NPH insulin, and ultralente human insulin and continuous subcutaneous infusion of insulin lispro. Diabetes 2000;49:2142-2148

107.Mullins P, Sharplin P, Yki-Jarvinen H, Riddle MC, Haring HU. Negative binomial meta-regression analysis of combined glycosylated hemoglobin and hypoglycemia outcomes across eleven Phase III and IV studies of insulin glargine compared with neutral

protamine Hagedorn insulin in Type 1 and Type 2 diabetes mellitus. Clin. Ther. 2007;29(8):1607–1619

108.Riddle MC, Rosenstock J, Gerich J. The treat-to-target trial: randomized addition of glargine or human NPH insulin to oral therapy of type 2 diabetic patients. Diabetes Care. 2003;26:3080–3086.

109.Gerstein HC, Bosch J, Dagenais GR, Diaz R, Jung H, Maggioni et al. ORIGIN Trial Investigators. Basal insulin and cardiovascular and other outcomes in dysglycemia. N Engl J Med 2012;367: 319-328

110.Havelund S, Plum A, Ribel U et al. The mechanism of protraction of insulin detemir: a long-acting, acylated analog of human insulin. Pharm Res 2004; 21(8): 1498–1504

111.Sørensen AR, Stidsen CE, Ribel U et al. Insulin detemir is a fully efficacious, low affinity agonist at the insulin receptor Diabetes Obes Metab 2010;12(8):665-673

112.Frier BM, Russell-Jones D, Heise T. A comparison of insulin detemir and neutral protamine Hagedorn (isophane) insulin in the treatment of diabetes: a systematic review. Diabetes Obes Metab 2013; 15(11): 978-986.

113.Porcellati F, Rossetti P, Busciantella NR et al. Comparison of Pharmacokinetics and Dynamics of the long-acting insulin analogs glargine and detemir at steady state in type 1 diabetes. Diabetes Care 2007;30:2447-2452

114. Lucidi P, Porcellati F, Rossetti P et al. Pharmacokinetics and Pharmacodynamics of therapeutic doses of basal insulins NPH, glargine and detemir after 1 week of daily administration at bedtime in type 2 diabetes. Diabetes Care 2011;34:1312-1314

115. Porcellati F, Lucidi P, Rossetti P et al. Differential effects of adiposity on Pharmacodynakics of basal insulins NPH, glargine, and

detemir in type 2 diabetes mellitus. Diabetes Care 2011;34:2521-2523

116.Rosenstock J, Davies M, Home PD, Larsen J, Koenen C, Schernthaner G. A randomised, 52-week, treat-to-target trial comparing insulin detemir with insulin glargine when administered as add-on to glucose-lowering drugs in insulin-naive people with type 2 diabetes. Diabetologia 2008; 51(3): 408-416.

117. Jonassen I, Havelund S, Hoeg-Jensen T, Steensgaard DB, Wahlund P O. Ribel U. Design of the novel protraction mechanism of insulin degludec, an ultra-long-acting basal insulin. Pharm Res 2012; 29(8): 2104–2114

118.Steensgaard DB, Schluckebier G, Strauss HM et al. Ligand-controlled assembly of hexamers, dihexamers, and linear multihexamer structures by the engineered acylated insulin degludec. Biochemistry 2013;52:295-309

119.Haahr H, Heise T. A review of the Pharmacological Properties of Insulin degludec and their clinical relevance. Clin Pharmacokinet 2014;53:787-800

120.Garber AJ, King AB, Del Prato S et al. Insulin degludec, an ultra-longacting basal insulin, versus insulin glargine in basal-bolus treatment with mealtime insulin aspart in type 2 diabetes (BEGIN Basal-Bolus Type 2): a phase 3, randomised, open-label, treat-to-target non-inferiority trial. Lancet 2012;379(9825):1498–1507.

121.Heller S, Buse J, Fisher M, Garg S et al. Insulin degludec, an ultra-longacting basal insulin, versus insulin glargine in basal-bolus treatment with mealtime insulin aspart in type 1 diabetes (BEGIN Basal-Bolus Type 1): a phase 3, randomised, open-label, treat-to-target non-inferiority trial. Lancet 2012;379(9825):1489–1497.

122.Marso SP, McGuire DK, Zinman B, Poulter NR, Emerson SS, Pieber TR et al Efficacy and Safety of Degludec versus Glargine in Type 2 diabetes. N Engl J Med 2017;377:723-732

123.Havelund S, Ribel U, Hubalek F, Hoeg-Jensen T, Wahlund PO, Jonassen Ib. Investigation of the physico-chemical properties that enable co-formulation of basal insulin degludec with fast-acting insulin aspart. Pharm Res 2015;32:2250-2258

124.Gough SC et al Insulin degludec/liraglutide (IDegLira) for the treatment of type 2 diabetes. Expert Rev Endocrinol Metab 2016;11:7-19

125.Becker RH, Dahmen R, Bergmann K, Lehmann A, Jax T, Heise T. New insulin glargine 300 Units /mL-1 provides a more even activity profile and prolonged glycemic control at steady state compared with insulin glargine 100 Units/mL-1. Diabetes Care 2015;38(4):637–643

126.Werner U, Tennagels N, Fanelli CG, Bolli GB. Equipotency of insulin glargine 300 and 100 U/mL with intravenous dosing but differential bioavailability with subcutaneous dosing in dogs. Diabetes Obes Metab 2021;23:166-174

127.Cheng A, Harris S, Giorgino F et al. Similar glycaemic control and less hypoglycaemia during active titration after insulin initiation with Gla300 vs IDeg-100: a subanalysis of the BRIGHT study. Diabetes Obes Metab. 2019; 22:346-354

128.Porcellati F, Lucidi P, Candeloro P et al. Pharmacokinetics, pharmacodynamics, and modulation of hepatic glucose production with insulin glargine U300 and glargine U100 at steady state with individualized clinical doses in type 1 diabetes. Diabetes Care 2019; 42:85–92

129.Rosenstock J, Cheng A, Ritzel R et al. More similarities than differences testing insulin glargine 300 U/mL versus insulin degludec

100 U/mL in insulin-naïve type 2 diabetes: The randomized head-to-head BRIGHT trial. Diabetes Care. 2018; 41:2147-2154

130.Philis-Tsimikas A, Klonoff DC, Khunto K, Bajaj HS, Leiter LA, Hansen MV et al. Risk of hypoglycaemia with insulin degludec versus insulin glargine U300 in insulin-treated patients with type 2 diabetes: the randomised, head-to-head CONCLUDE trial. Diabetologia 2020;63:698-710

131.Nishimura E, Kjeldsen T, Hubalek F et al. Molecular and biological properties of insulin Icodec, a new insulin analog designed to give a long half-life suitable for once-weekly dosing. Diabetes 2020:69(Suppl 1):236-OR

132.Rosenstock J, Bajaj HS, Janez AJ et al. Once-weekly insulin for type 2 diabetes without previous insulin treatment. N Engl J Med 2020;383:2107-2116

133. Moran-Thomas A. One hundred years of insulin for some. N Engl J Med 2021;385:293-295

……
……..

Legends for Figures and Tables:

Legends for the figures 1-10

Figure 1: Earlier researchers involved in the attempt to isolate insulin: George Ludwig Zülzer, Ernest Lyman Scott, Nicholae Paulescu and Israel Kleiner.

Figure 2: The four researchers involved in the discovery, and birth of insulin therapy: Frederick Grant Banting, John James Rickard Macleod and James Bertram Collip

Figure 3a: Frederick Sanger who was awarded the Nobel Prize in Chemistry in 1958, for his work on the structure of proteins, especially that of insulin.

Figure 3b: Rosalyn Yalow was awarded the Nobel Prize in Physiology or Medicine in 1977, for co-developing radio-immunoassay of insulin with Solomon Berson.

Figure 3c: Dorothy Hodgkin was awarded the Nobel Prize in Chemistry in 1964, for solving the physical (atomic) structure of molecules including insulin, using X-ray crystallography.

Figure 3d: Walter Gilbert and Frederick Sanger with Paul Berg, were awarded the Nobel Prize in Chemistry in 1980, for their contributions concerning the determination of base sequences in nucleic acids.

Figure 4: Percentage distribution of principal causes of death, among deceased people with diabetes in specified periods between 1898 and 1968 (Experience of Joslin Clinic, Boston. Mass, USA). Adapted from Marks and Krall (57).

Figure 5a: Plasma insulin and glucose concentrations in the morning and afternoon after subcutaneous (s.c.) injection of a mixture of regular and Lente insulin (admixed in the same syringe, ratio 20/80) 30 min before breakfast, in a group of people with T1DM. Note as a result of mealtime hypoinsulinemia and pre-lunch hyperinsulinemia, plasma glucose increases excessively post-meal, and decreases to hypoglycaemia values before lunch in T1DM. Adapted from Dimitriadis and Gerich (70)

Figure 4b**:** Plasma glucose and insulin concentrations overnight in a group of people with T1DM given regular and NPH insulin either admixed (ratio 45/55) at dinner (continuous line), or as separate injections of regular insulin at dinner and NPH insulin at bedtime (interrupted line). Adapted from Fanelli et al (71).

Figure 6: Stepwise biosynthesis of human insulin using recombinant DNA technology. Monnier et al (83).

The plasmid DNA of the protein to be cloned (A and B chains of human insulin or human proinsulin) are represented by dark blue quarter-circle arcs. After insertion in the plasmid vector (orange circles) of the host cells (bacteria or yeasts) the proteins, i.e. insulin chains (left part) or proinsulin (right part), are synthetized. Multiplication of the host cells results in replication of plasmid DNA of proteins and amplification of their production. The end protein products are recovered and after restoration of the disulfide bridges the biosynthetic human insulin is purified. The cell host chromosomic DNA is different from the plasmid DNA and is represented by folded green loops.

Figure 7: Primary structure of insulin, indicating the amino-acids involved in monomer to monomer interaction leading to dimers, dimer to dimer interaction leading to hexamers and those involved in receptor interaction to achieve biological effect. Adapted from Brange et al (91)

Figure 8: The structures of the original three rapid acting insulin analogues: insulin lispro, insulin aspart and insulin glulisine.

Figure 9: Absorption kinetics of the five different categories of intermediate and long-acting insulins (NPH, glargine 100 U/mL, detemir, degludec and glargine 300 U/mL) after injection in the subcutaneous tissue (SC tissue). Adapted from Monnier et al (83).

1. NPH insulin remains as crystals in the SC tissue before the release of insulin from the protamine insulin complex.

2. Insulin glargine which is soluble in the pharmaceutical preparation, is subject to micro-precipitation/crystallisation in the SC tissue at neutral pH, prior to dissolution into hexamers, dimers and finally monomers.

3. Insulin detemir is present as hexamers in the pharmaceutical preparation becoming di-hexamers in the SC tissue before dissociation into monomers which binds reversibly to albumin in the systemic circulation.

4.The insulin degludec is present as di-hexamers in the pharmaceutical preparation, becoming multi-hexamers in the SC tissue, followed by the extrusion of phenol and then zinc and dissociating into dimers and finally monomers, which bind reversibly to albumin in the systemic circulation.

5. The concentrated insulin glargine 300 U/mL is subject to micro-precipitation/crystallisation in the SC tissue. The SC depot is half the size of that depot observed with glargine 100 U/ml. There is slow dissolution into hexamers, dimers and monomers with the loss of ~20-30% bioavailability due to local enzymatic degradation (dark blue crosses) compared with glargine 100 U/ml. Note: For the acylated insulin preparations reversable binding to plasma albumin is represented as blue rectangles.

Figure 10: Structure of acylated insulins. Insulin detemir, degludec and icodec are acylated with a saturated fatty acid of increasing lengths, having removed the terminal B30 threonine. Insulins degludec and iodec are attached to a C16 and C20 fatty acid respectively by different spacers to the B29 amino acid. In addition, for icodec, 3 amino-acids substitutions have been made at positions A14, B16 et B25. Adapted from Monnier et al (83).

……
……..

Legends for Table 1-3

Table 1: Summary of the key milestones in the evolution of insulin preparations, and the accompanying scientific achievements during the period 1922-1980.

Table 2: Strategies for developing rapid-acting insulin analogues. Adapted from Brange and Volund (92)

Table 3: Summary of the major milestone in the development of recombinant DNA (rDNA) derived rapid, ultra-rapid and prolonged-acting acting insulin analogues during the period 1980-2021. Related scientific advancements in the use of recombinant DNA technology during the period 1977-2019 are highlighted.

……
……..