MOLECULAR
METABOLISM

Structural principles of insulin formulation and analog design: A century of innovation

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ABSTRACT

Background: The discovery of insulin in 1921 and its near-immediate clinical use initiated a century of innovation. Advances extended across a broad front, from the stabilization of animal insulin formulations to the frontiers of synthetic peptide chemistry, and in turn, from the advent of recombinant DNA manufacturing to structure-based protein analog design. In each case, a creative interplay was observed between pharmaceutical applications and then-emerging principles of protein science; indeed, translational objectives contributed to a growing molecular understanding of protein structure, aggregation and misfolding.

Scope of review: Pioneering crystallographic analyses—beginning with Hodgkin's solving of the 2-Zn insulin hexamer—elucidated general features of protein self-assembly, including zinc coordination and the allosteric transmission of conformational change. Crystallization of insulin was exploited both as a step in manufacturing and as a means of obtaining protracted action. Forty years ago, the confluence of recombinant human insulin with techniques for site-directed mutagenesis initiated the present era of insulin analogs. Variant or modified insulins were developed that exhibit improved prandial or basal pharmacokinetic (PK) properties. Encouraged by clinical trials demonstrating the long-term importance of glycemic control, regimens based on such analogs sought to resemble daily patterns of endogenous β-cell secretion more closely, ideally with reduced risk of hypoglycemia.

Major conclusions: Next-generation insulin analog design seeks to explore new frontiers, including glucose-responsive insulins, organ-selective analogs and biased agonists tailored to address yet-unmet clinical needs. In the coming decade, we envision ever more powerful scientific synergies at the interface of structural biology, molecular physiology and therapeutics.

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The centennial of insulin's discovery [1] represents the end of one era and the beginning of another [2]. The past century has seen transformational advances in molecular medicine at the intersection of basic science and clinical application. As described in the present series of articles in this special issue, investigations of insulin—extending from its sequence and structure to its formulation and delivery—have both heralded these advances and defined general paradigms in biophysics, structural biology, molecular genetics, integrative metabolism and pharmacology. In this review, we seek to highlight how these paradigms informed advances in insulin pharmacology and, conversely, how such applications have deepened our understanding of molecular principles. A central thread is provided by protein crystallization: in turn, as a vehicle for insulin's purification [3], formulation [3,4] and

structural analysis [5]. Landmark crystal structures of 2-Zn insulin hexamer (as determined at Oxford [6,7] and in Beijing [8—10]) stimulated the design of insulin analogs and underlay successive generations of biophysical studies, including studies by NMR spectroscopy [11—17] and cryo-EM single-particle image reconstruction [18—21] (for review, see [22] in this issue). We envisage that in the next decade, a continuing interplay between foundational and translational studies will enable new therapeutic approaches and treatment technologies for Type 1 and Type 2 diabetes mellitus (T1D and T2D) [23]. These frontiers promise to integrate protein engineering with systems biology and molecular physiology to address yet-unmet clinical needs [24,25]. Landmark clinical trials, such as the Diabetes Complications & Control Trial (DCCT [26] recently reviewed [27]) and its follow-up study (EDIC

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[28]), established the principle that rigorous glycemic control can prevent or delay microvascular complications (and perhaps macrovascular complications as well [29]). The goal of insulin replacement therapy (IRT) in T1D (and in a subset of patients with T2D) is to mimic, as closely as possible, the normal pattern of insulin secretion by the endocrine pancreas (Figure 1A). In healthy individuals, hormonal control of metabolism restricts excursions in blood glucose concentration (BGC) despite marked variation in the timing and extent of food intake and physical activity [30—32]. Because the time course of insulin action is critical to such regulation, molecular mechanisms of insulin self-association in a delivery device and of its disassembly in a subcutaneous (SQ) depot constrain the pharmacokinetic (PK) properties of insulin formulations (Figure 1B). In the first 50 years after the discovery of insulin, efforts focused on how best to formulate animal insulins (typically porcine and bovine insulin due to the abundance of pancreata from the food industry) [33]. These decades saw the development of both soluble formulations (such as "regular" insulin solutions comprising zinc hexamers), suspensions of amorphous precipitates (*semilente* zinc-insulin complexes) and microcrystalline suspensions (*ultralente* and neutral protamine Hagadorn; NPH) [3]. The general stabilizing effects of zinc ions and value of protamine (a basic DNA-binding peptide derived from sperm) were empirically discovered [34] in advance of their structural rationalizations (below) [35]. Early pharmaceutical development focused on long-acting products to (1) avoid the necessity of multiple daily injections, thereby enhancing patient convenience, and (2) mitigate the instability of insulin solutions, especially in the absence of zinc-mediated self-assembly. In addition, monitoring and optimization of prandial protocols were difficult in these years because technologies for measuring a patient's BGC (now routine [36,37]) were then limited. Accordingly, this review first focuses on structural determinants of insulin self-assembly [38] and its exploitation in an innovative series of basal [39,40] and rapid-acting products [41—43]. An overview of the respective time courses of

NPH and present basal insulin analog formulations is provided in Figure 1C.

1. INSULIN HEXAMER AND MODE OF ASSEMBLY

Insulin, the first protein with a known sequence [44] (for review, see [45,46]), also played a distinguished role in the history of protein crystallography. J.J. Abel's crystallization of zinc-free insulin in 1926 (the second protein to be crystallized after urease [47]), extended by Scott in 1934 to include zinc ions [48], set the stage for large-scale use of insulin crystallization in the pharmaceutical purification of animal insulins [49]. Additionally, x-ray diffraction studies of insulin crystals were undertaken by D. Crowfoot Hodgkin [50], who began in 1935 as a doctoral student at Cambridge University U.K. under the guidance of Prof. J.D. Bernal [51]. Techniques had not yet been developed to phase such diffraction patterns. After 35 years (and following methodological advances by M. Perutz and J.C. Kendrew in their respective crystallographic studies of hemoglobin [52] and myoglobin [53,54]), the Hodgkin laboratory determined an intermediate-resolution structure in 1969 [6] and high-resolution structure in 1971 [7]. This structure depicted the 2-Zn insulin hexamer, a dimer of trimers that each contained an axial zinc ion (bound to His^{B10} in octahedral coordination geometry). Designated T_6 in current nomenclature, the assembly could also be viewed as a trimer of zinc-free T_2 dimers (Figure 2A), a perspective in accordance with the relative extent of these self-association surfaces and with the stronger dimerization of insulin monomers in zinc-free solution. The dimer interface is largely nonpolar and contains a short antiparallel β-sheet (residues B24—B28 and its dimer-related mate); the two protomers in the dimer are similar in conformation but not identical. The mode of self-assembly of insulin as a dimer and zinc-coordinated trimer of dimers was striking for its structural elegance, prompting Blundell and colleagues to poetic allusion: "A thing of beauty is a joy forever," stated a celebrated figure
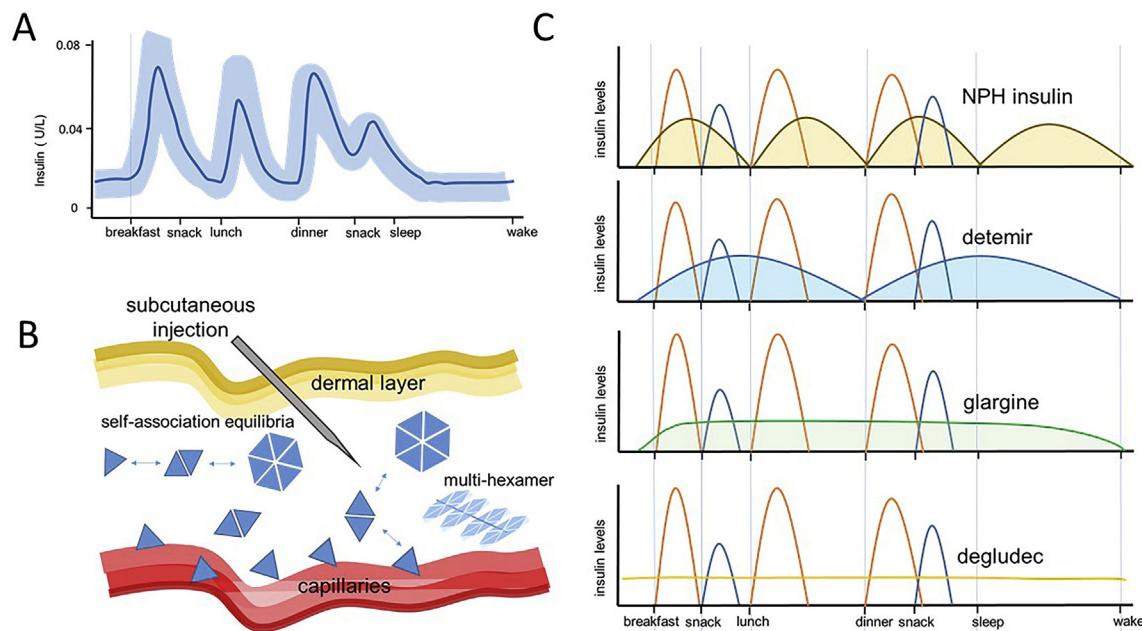
Figure 1: Physiological insulin secretion and pharmacologic principles. (A) Pattern of insulin concentrations in the blood of healthy people following meals (adapted from [260]). (B) Insulin self-association equilibrium within SQ space. Depending on analog formulation, insulin may form zinc hexamers (blue hexagons) or multi-hexamer assemblies; these dissociate to liberate zinc-free dimers, as well as monomers amenable to capillary absorption (red). (C) Schematic PK profiles (24 h cycle).
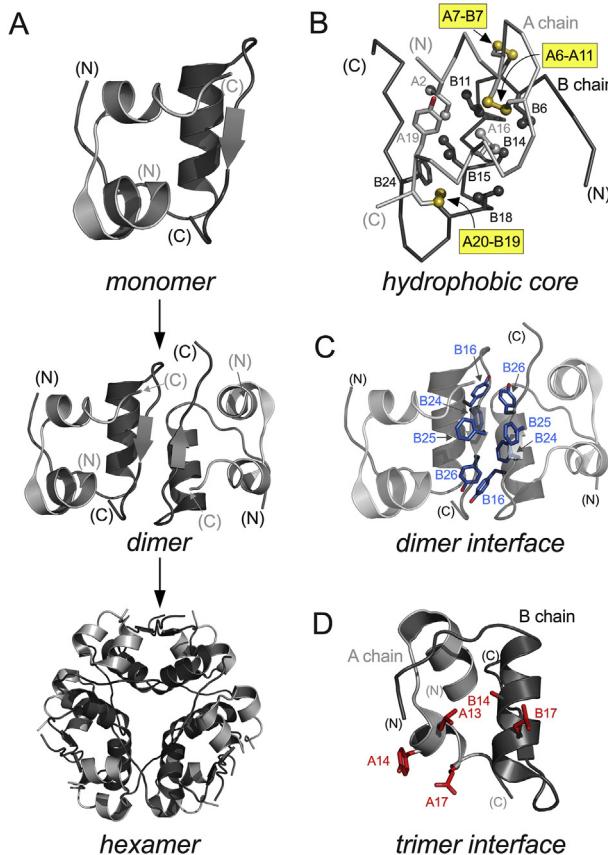
Figure 2: Insulin structure and self-assembly. (A) Self-association of monomer to dimer to hexamer. Color code: A chain, light gray ribbons; B chain, dark gray; (N) and (C) denote N- and C-termini of peptide chains. (B) Core packing of insulin monomer. Color code: A-chain residues (light gray), B-chain residues (dark gray). Sulfur atoms (gold spheres) and methyl groups (gray spheres) are shown at one-third van der Waals radius. Residues are labeled by sequence position. (C) Aromatic-rich dimer interface: residues B24—B26 and B16 are shown. Side chains (blue) are as labeled. (D) Residues at trimer surface are highlighted in red. Coordinates were obtained PDB entry 4INS (porcine insulin) [5].

caption (without explicit reference to Keats) [55]. The hydrophobic core of an insulin T-state monomer and its respective dimer and trimer interfaces are shown in Figure 2B—D. This structure contains elements now appreciated as features of globular proteins in general, including the canonical secondary structures α-helix, β-sheet and β-turn [56,57]. The crystal structure verified the disulfide pairing scheme originally defined by F. Sanger based on chemical methods [44], one on the protein surface (cystine B7-A7) and two within the protomer's core (cystines B19-A20 and A6—A11; yellow-shaded boxes in Figure 2B). It is of historical note that essentially the same structural features were elucidated in the years 1971—1974 by the Peking Insulin Structure Group under the leadership of D.C. Liang [8,9,58], a former trainee of Hodgkin [59].

Initial formulations of insulin in the 1920s were made in acidic solutions without zinc ions, conditions associated with rapid physical and chemical degradation [3]. Of special concern in formulation (as well as in the course of purification from pancreata) was fibrillation, aggregation-coupled misfolding to form β-sheet—rich polymers (amyloid; Figure 3A). The process of fibril formation, now regarded as a universal property of proteins as a class of heteropolymers [60,61], involves nucleation of misfolded "seeds" followed by rapid

propagation. Modern fibril-diffraction and cryo-EM studies of insulin fibrils have demonstrated close structural relationship between insulin fibrils and pathological deposits in human amyloidogenic diseases [62]. Beginning in the 1930s, empirical protection from fibrillation was achieved through the addition of zinc ions and/or the basic peptide *protamine* [63] to neutral solutions and microcrystalline suspensions, as described in turn:

(i) Lente formulations. Addition of zinc ions to solutions of insulin at pH 7.4 markedly reduces the protein's solubility, enabling preparation of intermediate or long-acting insulin formulations of an augmented shelf life. Amorphous zinc-insulin precipitates (*semilente*; intermediate) and microcrystalline zinc-insulin suspensions (*ultralente*; Figure 3B—D) were developed in the early 1950s by K. Hallas-Møller and coworkers at then—Novo Terapeutisk (now Novo Nordisk) [64,65]. The neutral-pH *ultralente* formulation achieved protracted action with once-a-day dosing. The marked pH-dependent kinetics of insulin crystallization necessitated a two-stage *ultralente* manufacturing process: initial crystals ($T_3R^f_3$ zinc hexamers; see below) grown at pH 5.5 (Figure 3E,F) were transferred to pH 7.4 with additional zinc ions and sodium chloride [3]. The resulting T_6 crystal structure of 2-Zn insulin provided a retrospective model for *ultralente* insulin as a basal microcrystalline suspension (Figure 3C,D) [66]. *Semilente* precipitates were obtained directly at pH 7.4. Binding of the antimicrobial preservative methylparaben was observed at dimer—dimer interfaces within the hexamers (space-filling ligands in Figure 3C), providing further stability. The complex phenomenology of insulin precipitation and crystallization in the *lente* series foreshadowed the discovery of the allosteric TR transition among zinc insulin hexamers [67,68] and current foundational interest in biophysical mechanisms by which protein crystals nucleate and grow [61,69].

(ii) Insulin—protamine complexes. In 1936, Hagedorn formulated the first intermediate-acting insulin formulation, designated *protamine insulinate* [70,71]. Protamine is a basic protein with isoelectric point >12 [72,73], and had been characterized earlier as a DNA-precipitating agent [74,75]. Sequences of vertebrate protamines are given in Figure 4A; the multiple Arg residues are highlighted in magenta. At the bottom of Figure 4A are fish sequences as used in clinical formulations [76]. Whereas *protamine insulinate* initially required a patient to mix an acidic solution of insulin with a neutral solution of protamines, Scott found that the addition of zinc ions at a low concentration led to a stable, neutral-pH formulation called *protamine zinc insulin* (PZI) in the form of amorphous or microcrystalline suspensions [34]. Absorption from an SQ depot continued for 1—3 days. Further improvements (including the addition of phenol or *meta*-cresol) were made by Krayenbuhl and Rosenberg in 1946 [77], leading to the development of Neutral Protamine Hagedorn (NPH) [78,79], a method of formulation that remains in current use. Manufacture of NPH is notable for its self-conversion of initial amorphous precipitates to crystals. Variation in the details of crystallization and components of the formulation were found to influence the duration of action [80]. More recently, *in vitro* SQ model systems have been developed to measure dissolution of insulin formulations [81] and correlate such data with *in vivo* (PK) profiles [82]. NPH suspensions are compatible with addition of soluble zinc-insulin hexamers to provide biphasic formulations whereby the soluble fraction provides rapid action and the microcrystalline fraction provides intermediate action [83,84]. The ratio of these components modulates the balance of these activities.
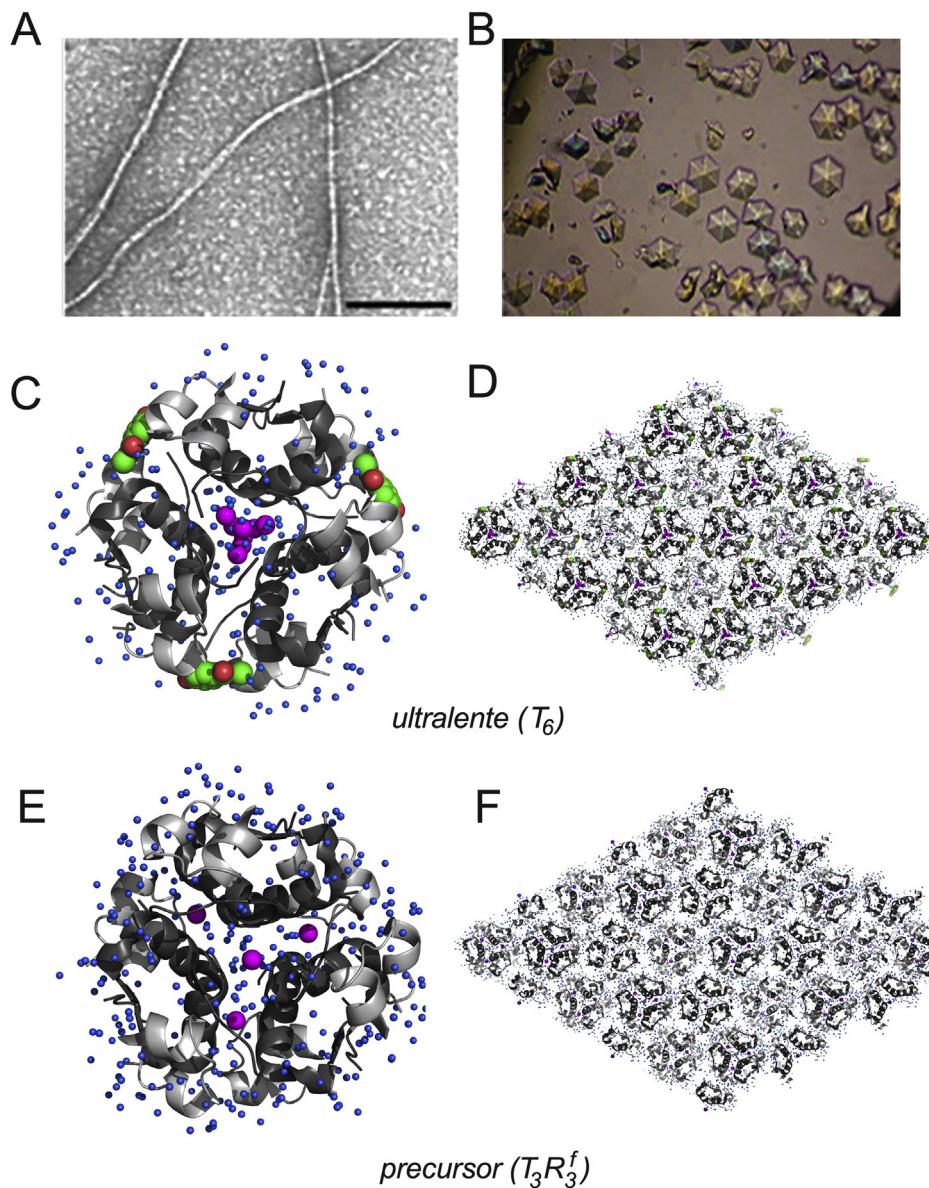
Figure 3: Insulin fibrillation and microcrystalline *ultralente* formulation. (A) Insulin fibrillation complicated initial acidic formulations of insulin and continues to pose a challenge at elevated temperatures [3,253]. EM photomicrograph of insulin fibrils (scale bar 200 nm; adapted from [257]). (B) Light microscopic image of *ultralente* crystals (panel adapted from [66]). (C) Cartoon representation of hexamer structure (T_6) of *ultralente* (PDB entry 2VK0). Color code: A chain, light gray ribbons; B chain, dark gray ribbons; zinc ions, magenta spheres; and water molecules, blue spheres with one-half van der Waals radii; methylparaben ligands are shown as CPK models in green (carbon) and red (oxygen). (D) Crystallographic arrangement of hexamers in a lattice filled with water (one-third van der Waals radii). (E) Crystal structure ($T_3R_3^f$) of *ultralente* precursor (PDB entry 2VJZ). Color code is as in panel C. (F) Crystallographic arrangement of hexamers of precursor lattice. Color code is as in panel D.

In this century, X-ray diffraction studies of NPH crystals revealed R_6 zinc insulin hexamers with bound phenolic ligands (Figure 4B) [35,85]. Electron density for protamine was diffuse or absent, even in co-crystals in which the insulin hexamers otherwise diffracted to high resolution. Use of Arg-rich model peptides (instead of protamine) suggested that binding may be in part within the solvent channel of the hexamer and in part between hexamers (possible locations of Arg residues are shown in magenta in Figure 4B). Although such crystallographic studies were motivated by potential insights pertinent to improving NPH-like formulations (such as through the possible use of protamine analogs or fragments), the absence of a specific mode of protamine binding has limited such efforts. NPH formulations of insulin continue to be useful and lower in cost than basal insulin analogs, even

though their PK profiles are shorter than basal analog formulations and exhibit a more pronounced peak. Indeed, the latter suboptimal features (together with the potential immunogenicity of fish protamines [86,87]) encouraged the development of basal insulin analogs.

2. BASAL INSULIN ANALOGS

Protamine-mediated precipitation and microcrystallization of insulin (like protamine-mediated precipitation of DNA) highlights the importance of electrostatic interactions—even when non-specific to structural mechanism—in modulating stability. Offsetting positive and negative charges, as introduced through the binding of heterologous peptides, may also be achieved within the insulin molecule. Thus

A *Sperm protamine P1 sequences*

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                 1        10        20        30        40        50
human        ARYRCCRSQSRSRYYRQRQRSRRRRRRSCQTRRRAMRCCRPRYRPRCRRH
bovine       ARYRCCLTHSGSRCRRRRRRCRRRRRRFGRRRRRRVCCRRYTVIRCTRQ
pig          ARYRCCRSHSRSRCRPRRRRCRRRRRRCCPRRRRRAVCCRRYTVIRCRRC
mouse        ARYRCCRSKSRSRCRRRRRRCRRRRRRCCRRRRRRCCRRRRSYTIRCKKY
horse        ARYRCCRSQSQSRCRRRRRRRCRRRRRRSVRQRRVCCRRYTVLRCRRRR
rabbit       VRYRCCRSQSRSRCRRRRRRRCRRRRRRCCQRRRVRKCCRRTYTLRCRRY
sheep        ARYRCCLTHSRSRCRRRRRRRCRRRRRRFGRRRRRRVCCRRYTVVRCTRQ
guinea pig   ARYRCCRSPSRSRCRRRRRRRFYRRRRRCHRRRRRRCCRRRYTRRCKRY

Rainbow trout  MPRRRRASRRVRRRRRRPRVSRRRRRGGRRRR
Chum salmon    PRRRRSSSRPVRRRRRRPRVSRRRRRRGGRRRR
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*insulin-protamine complex
(dihexamer)*

glargine dihexamers

*glargine hexamers
in a crystal lattice*
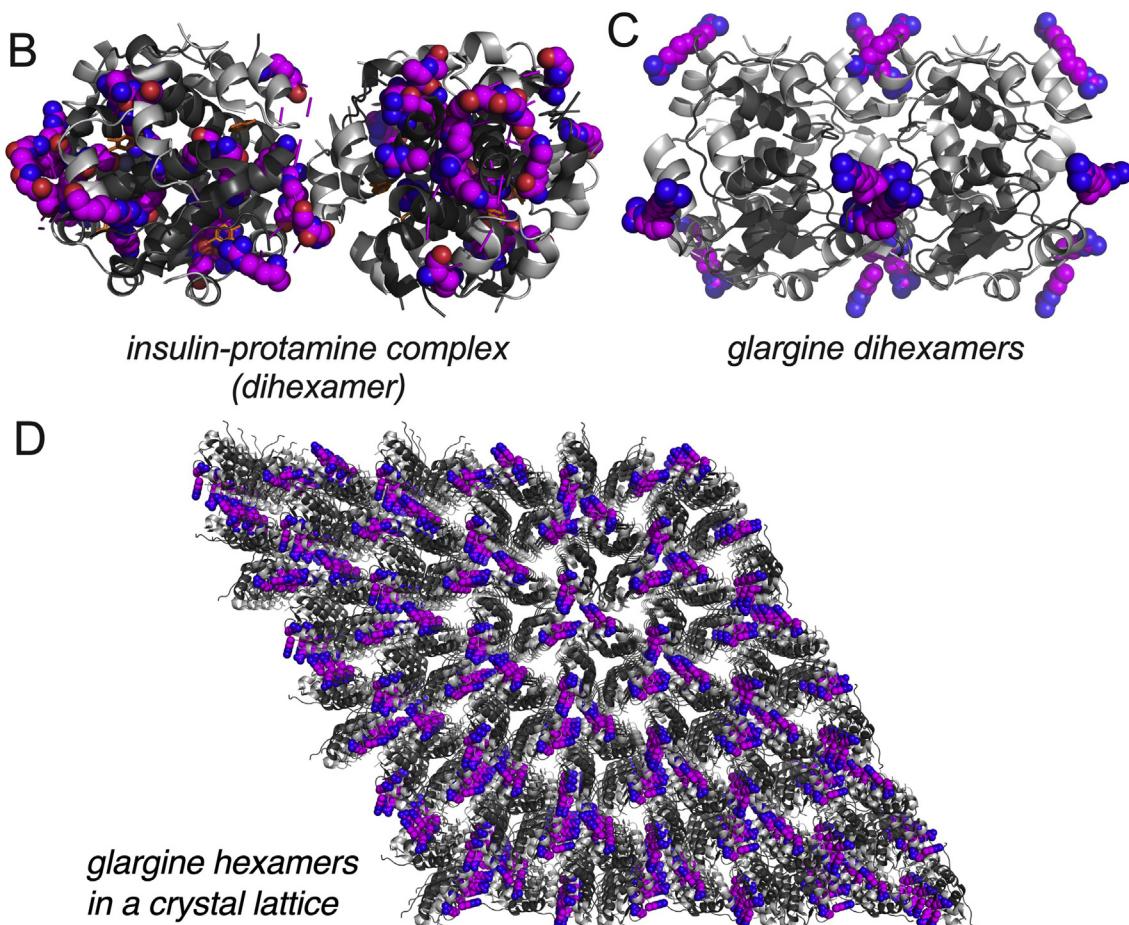
Figure 4: From NPH insulin to glargine as Arg-related basal analogs. (A) Protamine sequences of various species as Arg-rich peptides. Color code: conserved residues are in bold, Arg in magenta and hydroxyamino acid (conserved at position 8) in italics [258]. (B) Crystal structure of insulin−protamine complex is shown as dihexamers (PDB entry 7INS) [85]. Possible locations of Arg residues in highly disordered protamine peptide are shown as magenta spheres. (C) Molecular model of glargine forming Arg-mediated contacts between hexamers. Model was generated with PyMOL software using porcine insulin (PDB entry 4INS) as starting structure. (D) Array of glargine hexamers illustrating possible Arg-mediated crystal lattice.

emerged the principle of *isoelectric precipitation* of basal insulin analog formulations [78]. A property of wild-type insulin near its isoelectric point (pI ∼ 5.4), such precipitation in the SQ depot requires a shift in pI to neutrality. This principle was first demonstrated in NovoSol Basal, a recombinant analog containing substitution $Thr^{B27} \rightarrow Arg$ along with trypsin-catalyzed transamidation [88,89] (and hence neutralization) of the C-terminal carboxylate of Thr^{B30} [90]. With a net change of $+2$ in overall charge, the analog is soluble in an acidic formulation but insoluble at tissue pH; the insoluble SQ depot is further stabilized by zinc ions to promote hexamer assembly and fine-tune the assembly's net charge. Clinical development was discontinued due to reduced bioavailability of NovoSol Basal and local inflammatory reactions.
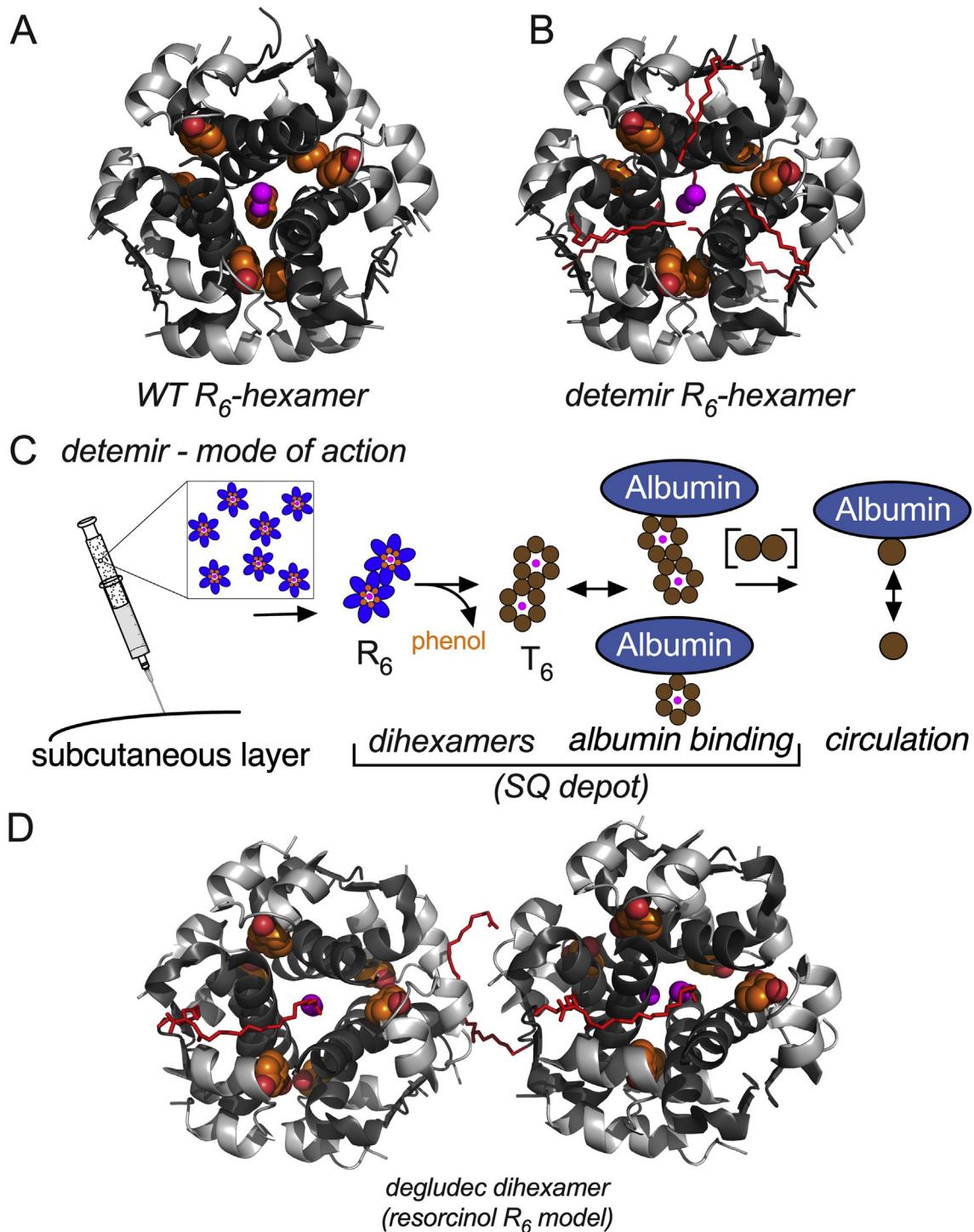
Figure 5: Acylated insulin analogs *detemir* and *degludec*. (A) Crystal structure of the wild-type R_6 hexamer (PDB entry 1ZNJ). Color code: A chain, light gray ribbons; B chain, dark gray ribbons; phenolic ligand, orange (CPK model); zinc ions, magenta spheres. (B) Crystal structure of R_6 *detemir* hexamer (PDB entry 1XDA) [97]. (C) Schematic representation of *detemir*'s acyl-stabilized SQ depot and reversible albumin binding in the circulation. Color code: R-state monomer, blue oval; T-state monomer, brown circles; bound phenol, orange circles; zinc ions, magenta circles. (D) Dihexamers in crystal structure of *degludec* (PDB entry 4AJX) [100]. This resorcinol-stabilized R_6-R_6 structure differs from the $R^f_3T_3$-$T_3R^f_3$ dihexamers (with abutting acyl-bridged T_3 trimers; coordinates not available) present in the vial under formulation conditions.

Exploitation of isoelectric precipitation [91] achieved broad clinical success through the development of insulin *glargine* (in Lantus® [92] and Toujeo® [93]). This analog, similar in concept to Novosol Basal, is formulated at pH 4 with zinc ions but without a buffer. The B chain is extended by two Arg residues (Arg^{B31} and Arg^{B32}) to likewise shift the pI with $+2$ change in net charge. Injection of the unbuffered formulation at pH 4 leads SQ precipitation at pH 7.4 to form a long-lived microcrystalline depot. A-chain substitution

Asn^{A21}→Gly circumvents acid-catalyzed chemical degradation of Asn^{A21} (which is well tolerated by the insulin receptor but immunogenic) and may augment hexamer stability [94]. Arg^{B31} and Arg^{B32}, which otherwise confer augmented mitogenicity [95], are cleaved by endogenous SQ exopeptidases. The predominant species absorbed into the bloodstream is hence Gly^{A21}-insulin. An analogy is suggested between the multiple Arg residues of protamine (Figure 4B) and the di-Arg element of insulin glargine (Figure 4C), in each case altering the net charge of the assembly and enhancing hexamer—hexamer interactions in a depot (as represented by a crystal lattice; Figure 4D). Lantus® is the most widely used long-acting insulin currently on the formulary. Increasing the formulated strength of *glargine* to U-300 (monomer concentration 1.8 mM) further prolongs absorption to provide, at steady-state, a near—peakless 24-hour profile with once-a-day dosing [96].

An unrelated mechanism of protracted action has been achieved by chemical modification rather than mutagenesis. The first such product (insulin *detemir* in soluble neutral-pH formulation Levemir®; Novo Nordisk) contains native-like R$_6$ zinc hexamers modified by myristic acid (Figure 5A,B; Figure 6A) [97]. Thr^{B30} is absent due to its method of recombinant manufacture. Acylation of insulin's unique side-chain amino group (of Lys^{B29}) in a zinc insulin analog hexamer both stabilizes the SQ depot and mediates binding to human serum albumin; the latter delays clearance from the bloodstream (Figure 5C) [98]. Direct linkage of the 14-carbon acyl group impairs receptor binding; in compensation, a U-100 formulation contains fourfold more protein molecules per mL than do standard U-100 formulations. Although this higher concentration may itself contribute to delayed SQ absorption, the duration of the SQ depot is nonetheless less than 18 h, leading to twice-a-day dosing, particularly in T1D. Remarkably, treatment with Levemir causes *less weight gain* than do other insulins [98], perhaps due to enhanced satiety-related hypothalamic signaling relative to the anabolic effects of insulin in the periphery [99]. Such preferential organ-specific signaling (if operative) would suggest a new biological frontier of next-generation insulin analog design (below).

Optimization of the acyl modification and interposition of a spacer element (glutamic acid) led to insulin *degludec* (Figure 5D; Figure 6B) [100]. This albumin-binding analog (the active component of Tresiba®; Novo Nordisk) provides a true and near—peakless 24-h formulation with once-a-day dosing. The neutral-pH solution contains a novel dimer of T$_3$R$^f_3$ zinc hexamers mutually bridged by a Glu-spaced-acyl modification at residue B29. A model is provided by the di-hexamer unit of an R$_6$ crystal structure (Figure 5D) [100]. Remarkably, the analog exploits insulin's TR transition (Figure 7A) in the SQ depot to undergo linear multi-hexamer assembly (Figure 7B). Extensive polymerization is triggered by a change in hexamer conformation (T$_3$R$^f_3$ → T$_6$) associated with release of the bound phenolic ligand [100]. This conformational change enables successive acyl-mediated contacts in a linear array of hexamers rather than the pairwise bridging observed in the formulated dimer of hexamers. A large-scale switch in state of assembly is coupled to the release of phenolic ligands from protein binding sites. This switch is unique to insulin *degludec* and represents the power of "molecular serendipity" beyond structure-based design.

Further optimization of the acylation strategy holds the promise of next-generation once-a-week insulins. Motivated by the success of once-a-week GLP-1 agonists [101—103], insulin Icodec® (Novo Nordisk) contains a longer and more complex adduct at position B29: a
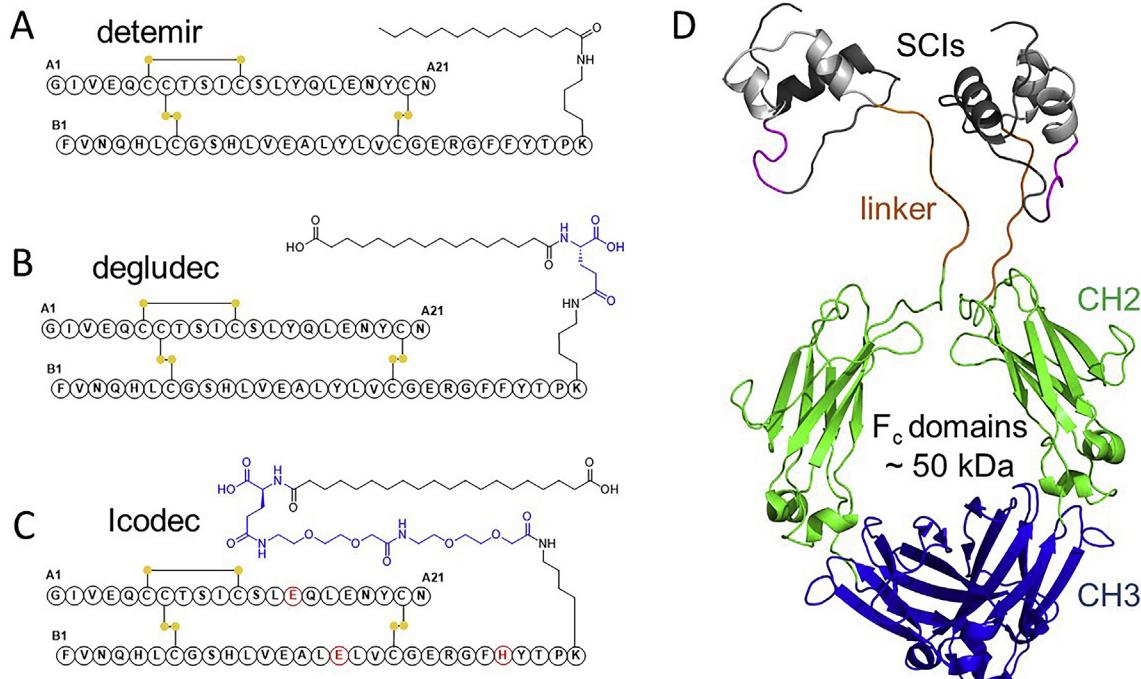
Figure 6: Long-acting insulin conjugates. (A) Insulin *detemir* contains an aliphatic C-14 group (myristic acid) on Lys^{B29} on a *des*-B30 scaffold. (B) Insulin *degludec* has a more complex acyl modification, hexadecanedioic acid with γ-L-glutamyl spacer (blue). (C) Icodec (an ultra-basal analog) is further modified at Lys^{B29} with a C-20 diacid group connected through a 2xOEG-gGlu linker (blue) and has amino acid substitutions (Tyr^{B16} → His, Phe^{B25} → His, and Tyr^{A14} → Glu, red) [104,106,111]. (D) Investigational basal insulin F$_c$ fusion protein (BIF; LY3209590) comprises of an SCI fused to a human IgG$_2$ F$_c$ domain; the insulin moiety contains modifications of Glu^{B16}, His^{B25}, Gly^{B27}, Gly^{B28}, Gly^{B29} and Gly^{B30} fused to a human IgG$_2$ F$_c$ domain through a tandem (Gly4-Gln) peptide linker (tan) at the A-chain C terminus (A21). This fusion protein offers once-weekly dosing [120].
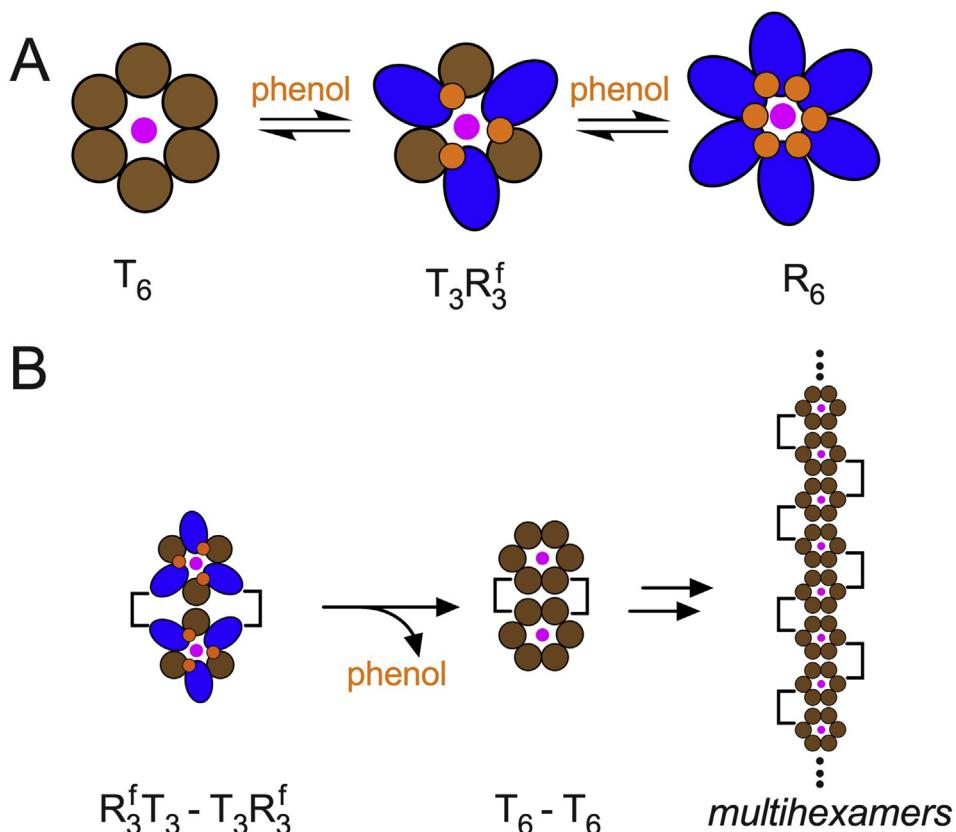
Figure 7: TR transition and protracted action of *degludec*. A) Schematic representation of the classical TR transition among zinc insulin hexamers. Color code: T-state monomer, brown circles; R-state monomer, blue oval; bound phenol, orange circles; zinc ions, magenta circles. B) Mechanism of protracted action of insulin *degludec* in Tresiba: under pharmaceutical formulations, the analog forms as $R_3^f T_3$-$T_3 R_3^f$ dihexamers [100], whereas SQ injection diffusion of phenol triggers a structural transformation, forming T_6-multihexamers as long-lived linear polymers. Brackets indicate acyl bridges between hexamers.

20-carbon di-acid linked through a composite linker (containing 2,2′-oxydiacetic acid and Glu; Figure 6C) [104]. Binding more tightly to serum albumin, the analog attains a maximum concentration 16 h after SQ injection with half-life 196 h [105,106]. Such an ultra-long PK profile is due not only to stronger albumin binding, but also to slower clearance by the insulin receptor: substitutions at positions B16 and B25 (Tyr$^{B16} \rightarrow$ His and Phe$^{B25} \rightarrow$ His) weaken receptor affinity [107—110] and also delay proteolysis [111]. The thermodynamic stability of the Icodec monomer is augmented by A-chain substitution Tyr-$^{A14} \rightarrow$ Glu. The risk of hypoglycemia with once-a-week insulin products will require careful assessment under real-world conditions.

A general platform for delaying the clearance of peptide and protein therapeutics is provided by macromolecular fusion [112,113]. One such approach employs unstructured polyethylene-glycol (PEG) moieties to enlarge the fusion's hydrodynamic radius and hence delay renal clearance. Application to insulin was investigated by the Lilly Research Laboratories (*peglispro*) [114—116]; clinical development was discontinued due to potential hepatic toxicity. A second approach exploits fusion to the F_c domain of immunoglobulins [117,118], assisted by F_c-receptor—mediated recycling [119]. Application to single-chain insulin (SCI) analogs has been described (Figure 6D) as being exemplified by basal insulin F_c (BIF®; Lilly LY3209590) [120]. Like Icodec, the insulin moiety contains paired B-chain substitutions (Tyr$^{B16} \rightarrow$ Glu and Phe$^{B25} \rightarrow$ His). Clinical testing in patients with T2D demonstrated a half-life of 17 days with near-peakless PK/PD profile for once-a-week dosing [120,121]. Alternative embodiments of this strategy have been described [122], including a heterodimeric two-chain SCI-F_c fusion [123].

3. PRANDIAL INSULIN ANALOGS

Even as prolonged activity was obtained by the above strategies, a continuing challenge was posed by the goal of rapid action—that is, comparable to that conferred by first-phase post-prandial pancreatic secretion in individuals without diabetes [124,125]. Pharmaceutical chemistry faces a Catch-22: the same self-assembly required to delay chemical and physical degradation (as observed in the 1920s in acidic solutions of insulin) delays absorption of the hormone from the SQ depot. So intractable seemed this molecular dilemma that alternative, more efficient routes of delivery were explored, including portal injection [126—128] and inhaled insulin [129—132], in each case seeking to exploit extensive peritoneal or pulmonary absorptive surfaces. Such engineering efforts are continuing, as exemplified by implanted intraperitoneal pumps [133,134] and inhalation devices for insulin powders [130]. Irrespective of technology, the central therapeutic goal was avoidance of immediate post-prandial hyperglycemia (from a delay in insulin action) and late post-prandial hypoglycemia (from a prolonged tail of insulin action). Recent progress in algorithm-based closed-loop systems [135—137], integrating insulin pumps with feedback from continuous glucose monitors (CGM), has further highlighted the need for ultra-rapid insulin formulations [138].
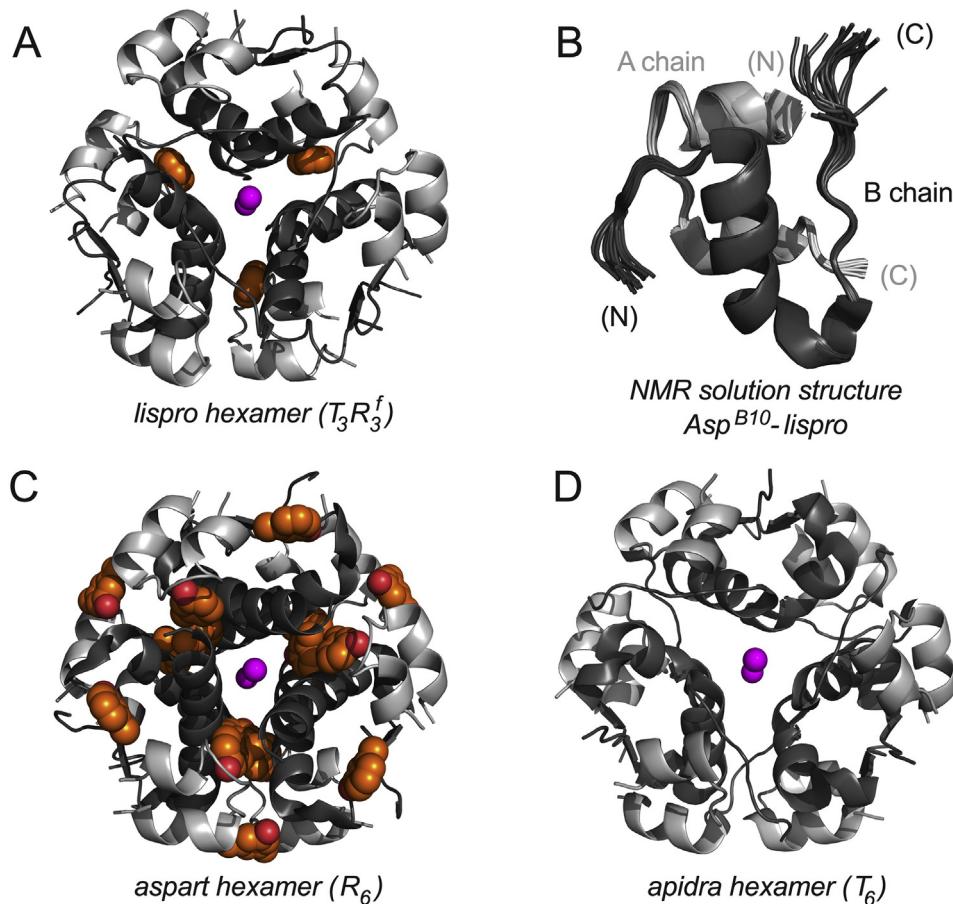
Figure 8: First-generation rapid-acting insulin analogs. (A) Crystal structure of *lispro* hexamer (Humalog and as made ultra-rapid in URLi; PDB entry 1LPH) [156]. Color code: A chain, light gray ribbons; B chain, dark gray ribbons; phenolic ligands, orange (CPK); zinc ions, magenta spheres. (B) NMR-derived solution structure of *lispro* analog containing Asp^{B10} (PDB entry 2JMN) [159]. N- and C-terminal residues are respectively labeled (N) and (C). (C) Crystal structure of insulin *aspart* as R$_6$-hexamer in presence of zinc ions and *meta*-cresol (Novolog and Fiasp; PDB entry 1ZEH) [157]. (D) *Glulisine* hexamer (Apidra; PDB entry 6GV0). Although zinc ions were not explicitly included in the crystallization buffer, apparently trace quantities enabled occupancy of the axial sites [161]; the clinical formulation is zinc-free as a means of accelerating absorption of analog monomers and dimers.

The essential problem lay in the size of the zinc insulin hexamer, too large for capillary absorption: its slow dissolution into zinc-free dimers and monomers imposed an intrinsic delay. The advent of insulin site-directed mutagenesis in 1979 [139,140] inspired efforts to circumvent this kinetic barrier via monomeric analog formulations [141]. An attractive candidate, pursued by Novo Nordisk, contained the substitution His$^{B10} \rightarrow$ Asp (X10 insulin) [142,143]. Originally identified as a mutation in proinsulin associated with a monogenic diabetes syndrome [144,145], Asp^{B10} stabilizes the zinc-free insulin monomer such that formulation is feasible in the absence of zinc ions and without further protein self-assembly. Indeed, in the absence of His at B10, axial coordination of zinc ions does not occur, and the negative charge of Asp^{B10} at the trimer interface precludes metal-ion—independent assembly. The substitution also enhanced the analog's affinity for the insulin receptor [142,146] (now appreciated to reflect introduction of a favorable salt bridge at the hormone—receptor interface [18,19]). Unfortunately, long-term rat testing of Asp^{B10}-insulin revealed an excess of mammary tumors [142,143]. Such carcinogenesis was subsequently shown to be mediated by enhanced binding to the mitogenic Type I IGF receptor (mimicking the homologous Glu^{B10} in IGF-I and IGF-II) and by a prolonged residence time on the insulin receptor [142]. Although the PK/PD properties of Asp^{B10}-insulin were appropriately accelerated, clinical development was curtailed. Beyond its immediate scientific interest, this experience demonstrated that the signaling properties of insulin are modifiable, thereby foreshadowing the goals of next-generation analog design (below).

Mitogenicities of rapid-acting insulin analogs in current clinical use are similar to that of wild-type insulin. Based on surveys of mutations at or near insulin's dimer interface [3,38,147], these analogs exhibit receptor-binding affinities indistinguishable from that of native human insulin and are amenable to pharmaceutical formulation. First-generation products insulin *lispro* (the active component of Humalog® and URLi®; Eli Lilly), insulin *aspart* (Novolog® and Fiasp®; Novo Nordisk) and insulin *glulisine* (Apidra®; Sanofi-Aventis) have proven safe and effective in multi-injection regimens and in pump use [148—150]. These analog formulations are nonetheless more susceptible to degradation above room temperature than corresponding human insulin formulations [151]. Current efforts focus on optimization of formulations (including the active excipients in URLi and Fiasp) to further accelerate SQ absorption [152—154]. Ultra-rapid analog formulations promise to enhance the robustness of feedback-based control algorithms in closed-loop systems [155]. Because insulin assembly ordinarily protects the hormone from degradation, these efforts encountered a tradeoff between more rapid action and formulation stability.

Crystal structures of insulin *lispro* (contained paired substitutions Pro^{B28}→Lys and Lys^{B29}→Pro) and *aspart* (Pro^{B28}→Asp) depicted native-like hexamer assembly with variant dimer interfaces (Figure 8A,C) [156,157]. Homogeneous hexamer assembly in each case requires specific binding of phenol or related ligand *meta*-cresol, classical antimicrobial agents. Unrelated to antimicrobial activity, phenolic ligands bind within zinc insulin hexamers to trigger allosteric reorganization: in this T→R transition, the R-state B chain forms an extended B1—B19 α-helix (or frayed B3—B19 α-helix; R^f) [157]. The crystal structure of insulin *lispro* (Figure 8A) was determined as a T$_3$R$^f_3$ zinc hexamer containing three bound phenolic ligands, and *aspart* as an R$_6$ hexamer containing six bound ligands (Figure 8C). The crystallographic T-state *lispro* protomer is similar to the solution structure of an isolated monomer (Figure 8B) [14,158,159]. Respective dimer interfaces retain native-like anti-parallel β-sheets (residues B24—B28) with only subtle structural distortions near the sites of substitution. Initial challenges to the pharmaceutical formulation of insulins *lispro* and *aspart* were posed by their increased susceptibility to degradation. The dual roles of phenolic ligands, serving both as antimicrobial preservatives and allosteric effectors of zinc hexamer assembly, proved key to the development of stable pharmaceutical formulations (Humalog and Novolog). The phenolic ligands rapidly diffuse upon SQ injection (presumably on a millisecond time scale [15]), enabling rapid hexamer disassembly and capillary absorption. Clinical development of these first-generation analog formulations thus exploited the molecular choreography of protein allostery, as first described in atomic detail by Hodgkin and colleagues [55,67,160]. Crystal structures of these analogs in turn illuminated the subtle role of a conserved proline (Pro^{B28}) at the native dimer interface [156].

Unlike Humalog and Novolog, Apidra® is a zinc-free formulation of an analog that retains Pro^{B28}: insulin *glulisine* (Asn^{B3}→Lys and Lys^{B29}→Glu). Net charge is decreased by one at neutral pH. Although the analog can form zinc hexamers with native-like dimer interfaces (Figure 8D) [161], its twin substitutions retard chemical and physical degradation, making hexameric assembly (and hence the need for zinc ions) unnecessary to achieve a stable pharmaceutical formulation. The U-100—strength protein solution (0.6 mM, as in U-100 WT formulations) contains an equilibrium mixture of zinc-free protein monomers and dimers, each capable of rapid absorption. To our knowledge, biophysical mechanisms by which paired substitutions Lys^{B3} and Glu^{B29} delay fibrillation in the absence of zinc ions are not well characterized. It is possible that electrostatic repulsion between distorted monomers delays formation of amyloidogenic seeds and their extension in protofilaments.

To enable insulin delivery even faster than first-generation analog products, an inhalable powdered form of insulin (Afrezza®) was developed, offering ultra-rapid onset (12 min) and short duration (3 h) [132]. Pulmonary delivery may be complicated by effects on lung tissue and perturbed by intercurrent respiratory infections. Efforts to obtain ultra-rapid PK parameters in an SQ analog formulation were motivated by the advent of closed-loop systems—an insulin pump controlled by an algorithm based on feedback from a CGM—as a strategy to optimize time in range (TiR) [138,155,162]. A variety of approaches have been considered (for review, see [163]). Absorption of SQ analog formulations may be accelerated by "active excipients," small molecules that enhance either hexamer disassembly or local blood flow [153]. Several reformulations of insulins *aspart* and *lispro* have received regulatory approval: Fiasp® [164] and URLi® (in US) or Lyumjev® (in Europe), respectively [165,166]. Clinical studies have provided evidence that URLi is superior to Humalog with respect to post-prandial glycemic control in patients with T2D [167] or T1D [168].

BCLIS® is an investigational ultra-rapid reformulation of *lispro* containing citrate and BC222, a novel oligomeric excipient [169] with PK properties similar to Fiasp® [170]. Additional investigational strategies to stabilize ultra-fast insulin formulations have focused on surfactants and amphiphilic polymers to modify the monomer—air—liquid interface to prevent fibrillation interfacial denaturation. Progress has been made—for example, through high-throughput screening of synthetic acrylamide-based host—guest copolymers [171,172].

4. NEXT-GENERATION INSULIN ANALOGS

Current frontiers of insulin analog design extend beyond pharmacokinetics to consider broad categories of unmet medical needs. The most immediate seeks molecular embodiment of a closed-loop system, whereby the feedback provided by a CGM is instead provided by features of the insulin analog or its macromolecular interactions. Such *glucose-responsive insulins* (GRIs) thus define a major and long-standing goal in molecular pharmacology (for recent reviews, see [173—175]. Intrinsic (or unimolecular) GRIs envisage a modified hormone whose bioavailability or activity is glucose-dependent [25]. Potential technologies include sequestration of the insulin analog within a glucose-dependent SQ depot or circulating inactive complex, released under conditions of hyperglycemia. A wide variety of endogenous macromolecules (such as those in interstitial spaces, endothelial cells or components of blood) might in principle be exploitable as glucose-responsive carrier proteins or cellular clearance systems [113]. The following schemes exploit chemical or protein-based glucose sensing. The simplest class of unimolecular GRIs contain chemical glucose sensors, such as *phenylboronic acid* (PBA) [176—178]. Such schemes may involve a polymer-based release system [173,174], an endogenous carrier such as albumin or an endogenous lectin [179]. Diboronate-based glucose sensors have also been employed to confer glucose-sensitive binding to albumin [180], in principle more selective than PBA for glucose relative to other monosaccharides [181,182]. A variety of endogenous carrier proteins (beyond albumin) offer promising technologies [113,183], including glucose transporter GLUT-1 [184—186]. In such schemes, insulin is linked to a glucose-displaceable carrier-binding element. Although these mechanisms are ordinarily reversible (with excursions in BGC), an interesting elaboration involved irreversible glucose-dependent clearance: clinical trials undertaken by Merck investigated a saccharide-modified insulin whose baseline clearance by endogenous mannose receptors was attenuated under hyperglycemic conditions [187]. Although partial GRI activity was observed in animal models, efficacy was limited in human subjects. The simplest unimolecular GRI would be self-contained and so function without an endogenous or exogenous macromolecular partner. This principle was recently illustrated through design of a fructose-dependent insulin analogue [188] based on insulin's native hinge [189]. Opening and closing of this hinge under the control of a monosaccharide is coupled to allosteric reorganization of the insulin receptor [188] and hence transmembrane signalling [22].

A GRI-like therapeutic strategy has been proposed which circumvents the need for a chemical glucose sensor. This approach exploits an endogenous switch in hepatic physiology: insulin opposes glucagon activity under hyperglycemic conditions, while hypoglycemia enhances glucagon signaling, mitigating insulin's otherwise inhibitory effect on hepatic glucose output [190—192]. Cherrington and colleagues have reported that concurrent injection of both hormones both provides glycemic control and buffers hypoglycemia. Encouraging clinical results were presented at the 80th ADA meeting [193]. Related studies by Novo Nordisk (using different insulin/glucagon ratios) provided

evidence that such dual hormone therapy can not only mitigate risk of hypoglycemia, but also insulin-induced weight gain [194,195]. Because such co-administration exploits a physiologic switch in the liver, the need for chemical glucose sensors or a glucose-dependent carrier (or clearance system) is circumvented.

Organ-specific insulins. Mouse models exhibiting organ-specific knockout of the insulin receptor (*IR*) or its substrates (*IRS* genes) have delineated respective physiological contributions of organ-specific insulin signaling [196—198]. These data have deepened long-standing concerns regarding peripheral administration of insulin versus pancreatic secretion into portal circulation [199]. Hepatoselective insulin analogs have long been sought to mimic physiological first-pass hepatic metabolism to overcome relative "under-insulinization" of the liver with suboptimal suppression of hepatic gluconeogenesis and relative "over-insulinization" of peripheral tissues; these imbalances in standard SQ insulin replacement therapy may exacerbate insulin resistance and contribute to weight gain [199]. Partial hepatic targeting was achieved by *peglispro* (see above), to which is ascribed its larger hydrodynamic radius: the PEG moiety impaired access to peripheral capillaries, relative to the more expansive sinusoids of the liver [200]. It is not known whether the hepatoselectivity of this analog is related to risk of hepatic injury as unexpectedly observed in clinical trials.

Brain-selective insulin analogs would be of interest, as insulin can regulate food intake [201,202] as a neuroendocrine hormone [203,204]. Insulin-regulated pathways have been defined in the amygdala and hypothalamus [205]. Proof of principle has been suggested by studies of insulin *detemir*, as its SQ administration leads to higher brain signaling relative to human insulin [206]. Although molecular mechanisms are not well understood, it is possible that the acylated analog exhibits enhanced transport through the blood—brain barrier. As a class, acylated analogs (Levemir® and Tresiba®) may thus reduce food intake and mitigate weight gain relative to non-acetylated analogs, such as insulin *glargine* [207]. Binding to albumin (the most abundant protein in the cerebral spinal fluid) may promote CNS activity, including therapeutic benefits in Alzheimer's disease [99,208]. Because progressive weight gain and cognitive impairment are of overarching concern (particularly in T2D), augmented insulin signaling in the brain would be of broad interest. Given that the IR glycoform mass in the brain differs from that in peripheral tissues [209], specific receptor targeting may be obtained unrelated to albumin binding [210].

Insulin signaling in adipose tissue underlies lipid storage and contributes to overall glucose homeostasis [198]. Targeting the adipocyte, seemingly a paradoxical goal in relation to lipotoxicity and insulin resistance, is nonetheless of investigational interest in relation to novel adipokines [211]. In mice, for example, palmitoleic acid inhibits lipogenesis and increases insulin sensitivity in liver and skeletal muscle [212]. Branched esters of hydroxylated fatty acids can also elicit anti-beneficial effects on glucose tolerance [213]. A putative adipocyte-selective insulin analog might also suppress synthesis and secretion of sphingolipid C16:0 ceramide, a mediator of insulin resistance [214] as affected by CerS6-specific antisense oligonucleotides [215].

Biased signaling. Biased signaling (or "biased agonism") differentially modulates a receptor's various signaling outputs. Such bias (due to subtle conformational differences among receptor—ligand complexes) has been shown in studies of G-protein—coupled receptors (GPCRs) [216], including the β-adrenergic receptor [217,218], opioid receptor [219] and chemokine receptor CXCR3 [220]. Biased activation of the insulin receptor was envisioned by Brown and Goldstein as a

mechanism to mitigate *selective hepatic insulin resistance* in the pathogenesis of hepatic steatosis [221—223]. A hallmark of T2D is the impairment of insulin signaling mediating glycemic control while excess signaling continues to drive lipid biosynthesis [224] and mitogenicity [225]. A liver-targeted biased agonist of the insulin receptor could therefore be transformational. The plausibility of such analogs has been suggested by phosphoproteomic analysis of insulin signaling [226,227]. Receptor-targeted phage display peptides, for example, bind at or near insulin's native receptor-binding sites but stimulate a distinct pattern of signaling outputs [228—230]. Yeast display technology may enable this approach to generalize to libraries of insulin variants [231—233] as demonstrated by Chou and colleagues [234]. The concept of biased agonism may also be exploited to reduce the baseline mitogenicity of insulin, of interest in relation to epidemiological relationships between T2D and several common cancers [235—237]. Mitogenic signaling by insulin analogs has therefore been intensively investigated [238—240]. Insight has been obtained from comparative studies of insulin-like growth factors (IGF-I and IGF-II) [241]. Insulin analogs differ in their extent of cross-binding to the mitogenic Type 1 IGF receptor (IGF-1R) and relative affinities for the two splicing isoforms of the insulin receptor (IR-A and IR-B).

5. CO-ADMINISTRATION OF INSULIN WITH OTHER HORMONES

Concurrent or co-administration of insulin with other peptide hormones may be of therapeutic benefit to enhance long-term glycemic control while reducing risk of hypoglycemia or weight gain. An example is provided by combination therapy with insulin and a glucagon-like peptide-1 receptor agonist (GLP-1RA) in T2D [242,243]. FDA-approved titratable fixed-ratio combinations formulate a basal insulin with a short-acting GLP-1RA [244—246]. Concurrent therapy with long-acting GLP-1RAs (recently reviewed [103]) suggest therapeutic advantages over short-acting GLP-1RAs [247]. Once-a-week basal insulin analog formulations presently in development (see above) promise to enable synchronous co-administration or various fixed-ratio combinations with once-a-week GLP-1RAs. Combination of insulin analogs with other hormones are also of interest. In T1D, for example, co-administration with pramlintide (an amylin analog), recapitulating endogenous β-cell co-secretion, promises to mitigate weight gain and hypoglycemic risk via increased satiety, delayed gastric emptying and inhibition of glucagon secretion [248,249]. Although co-administration of pramlintide is complicated by the incompatibility of its formulation with insulin, an investigational dual-hormone pump has been shown to increase TiR and reduce hypoglycemic events [250]. A novel co-formulation of insulin and pramlintide has also been described [172]. Co-injection of insulin and glucagon is discussed above as an approach to mitigating hypoglycemic risk without the need for engineered glucose sensing.

6. CONCLUDING REMARKS

The centennial of insulin's discovery in Toronto and concurrent 50th anniversary of its high-resolution crystal structure's determination in Oxford [6,7] and in Beijing [8—10] provide an opportunity to celebrate a remarkable history of synergy between basic science and clinical translation. Banting's medical background (as a surgeon at the Western front in World War I) motivated near-immediate use of the initial insulin solutions in patients moribund in accordance with the natural history of untreated T1D [1]. Development of insulin formulations preceded and encouraged the establishment of foundational

principles of protein science, including molecular mechanisms of self-assembly, crystallization, degradation and fibrillation [3]. In each of these subjects, studies of insulin aimed to define paradigms of subsequent general application [2]. It is remarkable the extent to which empirical advances in formulation chemistry anticipated, in some cases by decades, their structural elucidation and mechanistic understanding. Celebrated examples include zinc-mediated self-assembly, phenol-stabilized microcrystallization and the electrostatics of protamine—insulin complexes [35,66,85]). This history combines careful experimentation with serendipity, beginning in the years immediately after the landmark events in Toronto in 1921—1922 [259]. The phenomenology of the rhombohedral transition among zinc-insulin crystals in the 1950s ultimately led to high-resolution characterization of the allosteric TR transition as a model for the long-range transmission of conformational change in protein assemblies [56]. Such transmission, extended in recent years to the hormone—receptor complex [18—21], underlies the mechanism of insulin signaling (for review, see [22] in this issue).

Near-term therapeutic goals envision a transition from rapid-acting analog formulations to ultra-rapid [152,164—166], and from "peakless" once-a-day basal formulations to ultra-basal with once-a-week dosing [106,251]. Next-generation insulins seek to transcend pharmacokinetics (however significant) in four directions. First, "smart" insulin analogs or molecular systems promise to enhance glycemic control by reducing the risk of hypoglycemia and the need for stringent patient self-monitoring [252]. Second, ultra-stable insulin products promise to circumvent the complex and costly cold chain of insulin transport, storage and use in underdeveloped regions of the world—a key barrier given the growing pandemic of diabetes [253,254]. Third, organ-selective insulin analogs (such as to the liver or brain) may address unintended side consequences of insulin replacement therapy [255,256]. Finally, systems-level dissection of branching post-receptor insulin signaling pathways may enable the design of biased agonists to favor balanced homeostatic regulation. Combinations of these molecular technologies with other hormones (such as GLP-1 receptor agonists and amylin analogs) may yield therapeutic synergies. Prospects for continued innovation in insulin's second century are bright.

DISCLOSURE

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CONFLICT OF INTEREST

None declared.

REFERENCES

[1] Bliss, M., 2019. The discovery of insulin, 25th Anniversary Edition. University of Toronto Press.

[2] Flier, J.S., Kahn, C.R., 2021. Insulin: a pacesetter for the shape of modern biomedical science and the nobel prize. Molecular Metabolism, 101194.

[3] Brange, J., 1987. Insulin preparations. Galenics of insulin. Springer. p. 17—73.

[4] Jen, A., Merkle, H.P., 2001. Diamonds in the rough: protein crystals from a formulation perspective. Pharmaceutical Research 18(11):1483—1488.

[5] Baker, E.N., Blundell, T.L., Cutfield, J.F., Dodson, E.J., Dodson, G.G., Hodgkin, D.M.C., et al., 1988. The structure of 2-Zn pig insulin crystals at 1.5 Å resolution. Philosophical Transactions of the Royal Society B 319(1195):369—456.

[6] Adams, M.J., Blundell, T.L., Dodson, E.J., Dodson, G.G., Vijayan, M., Baker, E.N., et al., 1969. Structure of rhombohedral 2 zinc insulin crystals. Nature 224(5218):491—495.

[7] Blundell, T., Cutfield, J.F., Cutfield, S.M., Dodson, E.J., Dodson, G.G., Dodson, E.J., et al., 1971. X-ray analysis and the structure of insulin. Nature 231(5304):506—511.

[8] Peking Insulin Structure Group, 1971. Peking Review 40. p. 11—6.

[9] Peking Insulin Structure Group, 1975. Scientia Sinica 17. p. 752—78. S.

[10] Wang, J.-H., 1998. The insulin connection: dorothy Hodgkin and the Beijing insulin group. Trends in Biochemical Sciences 23(12):497—500.

[11] Hua, Q., Weiss, M.A., 1991. Comparative 2D NMR studies of human insulin and despentapeptide insulin: sequential resonance assignment and implications for protein dynamics and receptor recognition. Biochemistry 30(22):5505—5515.

[12] Knegtel, R.M., Boelens, R., Ganadu, M.L., Kaptein, R., 1991. The solution structure of a monomeric insulin: a two-dimensional ^{1}H-NMR study of des-(B26—B30)-insulin in combination with distance geometry and restrained molecular dynamics. European Journal of Biochemistry 202(2):447—458.

[13] Sørensen, M.D., Led, J.J., Sørensen, O.W., 1994. A new 2D NMR method for measurement of J_{HH} coupling constants. Journal of Biomolecular NMR 4(1):135—141.

[14] Olsen, H.B., Ludvigsen, S., Kaarsholm, N.C., 1996. Solution structure of an engineered insulin monomer at neutral pH. Biochemistry 35(27):8836—8845.

[15] Jacoby, E., Hua, Q.X., Stern, A.S., Frank, B.H., Weiss, M.A., 1996. Structure and dynamics of a protein assembly. ^{1}H-NMR studies of the 36 kDa R_6 insulin hexamer. Journal of Molecular Biology 258(1):136—157.

[16] Hua, Q.-x., Xu, B., Huang, K., Hu, S.-Q., Nakagawa, S., Jia, W., et al., 2009. Enhancing the activity of a protein by stereospecific unfolding: conformational life OF insulin and its evolutionary origins. Journal of Biological Chemistry 284(21):14586—14596.

[17] Hua, Q.-X., Jai, W., Weiss, M.A., 2011. Conformational dynamics of insulin. Frontiers in Endocrinology 2:48.

[18] Scapin, G., Dandey, V.P., Zhang, Z., Prosise, W., Hruza, A., Kelly, T., et al., 2018. Structure of the insulin receptor-insulin complex by single-particle cryo-EM analysis. Nature 556(7699):122—125.

[19] Weis, F., Menting, J.G., Margetts, M.B., Chan, S.J., Xu, Y., Tennagels, N., et al., 2018. The signalling conformation of the insulin receptor ectodomain. Nature Communications 9(1):1—10.

[20] Uchikawa, E., Choi, E., Shang, G., Yu, H., Bai, X.-c., 2019. Activation mechanism of the insulin receptor revealed by cryo-EM structure of the fully liganded receptor-ligand complex. eLife 8:e48630.

[21] Gutmann, T., Schäfer, I.B., Poojari, C., Brankatschk, B., Vattulainen, I., Strauss, M., et al., 2020. Cryo-EM structure of the complete and ligand-saturated insulin receptor ectodomain. Journal of Cell Biology 219(1):e201907210.

[22] Lawrence, M.C., 2021. Understanding insulin and its receptor from their three-dimensional structure. Molecular Metabolism. https://doi.org/10.1016/j.molmet.2021.101255.

[23] American Diabetes Association, 2021. Diabetes care in the hospital: standards of medical care in diabetes. Diabetes Care 44(Supplement 1):S211—S220.

[24] Zaykov, A.N., Mayer, J.P., DiMarchi, R.D., 2016. Pursuit of a perfect insulin. Nature Reviews Drug Discovery 15(6):425—439.

[25] Jarosinski, M.A., Dhayalan, B., Rege, N., Chatterjee, D., Weiss, M.A., 2021. 'Smart' insulin-delivery technologies and intrinsic glucose-responsive insulin analogues. Diabetologia, 1016—1029.

[26] The diabetes control and complications trial/epidemiology of diabetes interventions and complications (DCCT/EDIC) Study Research Group, 2005. Intensive diabetes treatment and cardiovascular disease in patients with type 1 diabetes. New England Journal of Medicine vol. 353(25):2643—2653.

[27] Bebu, I., Braffett, B.H., Orchard, T.J., Lorenzi, G.M., Lachin, J.M., Group, D.E.R., 2019. Mediation of the effect of glycemia on the risk of CVD outcomes in type 1 diabetes: the DCCT/EDIC study. Diabetes Care 42(7):1284—1289.

[28] Cleary, P.A., Orchard, T.J., Genuth, S., Wong, N.D., Detrano, R., Backlund, J.-Y.C., et al., 2006. The effect of intensive glycemic treatment on coronary artery calcification in type 1 diabetic participants of the Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications (DCCT/EDIC) Study. Diabetes 55(12):3556—3565.

[29] Nathan, D.M., 2021. Realising the long-term promise of insulin therapy: the DCCT/EDIC study. Diabetologia 64(5):1—10.

[30] Polonsky, K., Given, B., Van Cauter, E., 1988. Twenty-four-hour profiles and pulsatile patterns of insulin secretion in normal and obese subjects. Journal of Clinical Investigation 81(2):442—448.

[31] Nuttall, F.Q., Gannon, M.C., 1991. Plasma glucose and insulin response to macronutrients in nondiabetic and NIDDM subjects. Diabetes Care 14(9):824—838.

[32] Sheard, N.F., Clark, N.G., Brand-Miller, J.C., Franz, M.J., Pi-Sunyer, F.X., Mayer-Davis, E., et al., 2004. Dietary carbohydrate (amount and type) in the prevention and management of diabetes: a statement by the American Diabetes Association. Diabetes Care 27(9):2266—2271.

[33] Deckert, T., 1980. Intermediate-acting insulin preparations: NPH and lente. Diabetes Care 3(5):623—626.

[34] Scott, D., Fisher, A., 1936. Studies on insulin with protamine. Journal of Pharmacology and Experimental Therapeutics 58(1):78—92.

[35] Norrman, M., Hubálek, F., Schluckebier, G., 2007. Structural characterization of insulin NPH formulations. European Journal of Pharmaceutical Sciences 30(5):414—423.

[36] Chehregosha, H., Khamseh, M.E., Malek, M., Hosseinpanah, F., Ismail-Beigi, F., 2019. A view beyond HbA_{1c}: role of continuous glucose monitoring. Diabetes Therapy 10(3):853—863.

[37] Olansky, L., Kennedy, L., 2010. Finger-stick glucose monitoring: issues of accuracy and specificity. American Diabetes Association.

[38] Dodson, G., Dodson, E., Turkenburg, J., Bing, X., 1993. Molecular recognition in insulin assembly. Biochemical Society Transactions 21(3):609—614.

[39] Zinman, B., 2013. Newer insulin analogs: advances in basal insulin replacement. Diabetes, Obesity and Metabolism 15(s1):6—10.

[40] Battelino, T., Edelman, S.V., Nishimura, R., Bergenstal, R.M., 2021. Comparison of second-generation basal insulin analogs: a review of the evidence from continuous glucose monitoring. Diabetes Technology & Therapeutics 23(1):20—30.

[41] Brange, J., 1997. The new era of biotech insulin analogues. Diabetologia 40(2):S48—S53.

[42] Birnbaum, D.T., Kilcomons, M.A., DeFelippis, M.R., Beals, J.M., 1997. Assembly and dissociation of human insulin and Lys^{B28}, Pro^{B29}-insulin hexamers: a comparison study. Pharmaceutical Research 14(1):25—36.

[43] Wilson, L.M., Castle, J.R., 2020. Recent advances in insulin therapy. Diabetes Technology & Therapeutics 22(12):929—936.

[44] Brown, H., Sanger, F., Kitai, R., 1955. The structure of pig and sheep insulins. Biochemical Journal 60(4):556—565.

[45] Sanger, F., 1959. Chemistry of insulin. Science 129(3359):1340—1344.

[46] Stretton, A.O., 2002. The first sequence: fred Sanger and insulin. Genetics 162(2):527—532.

[47] Abel, J.J., 1926. Crystalline insulin. Proceedings of the National Academy of Sciences 12(2):132—136.

[48] Scott, D.A., 1934. Crystalline insulin. Biochemical Journal 28(4):1592—1602, 1591.

[49] Lewis, G.F., Brubaker, P.L., 2021. The discovery of insulin revisited: lessons for the modern era. Journal of Clinical Investigation 131(1):e142239.

[50] Vijayan, M., 2002. The story of insulin crystallography. Current Science 83(12):1598—1606.

[51] Crowfoot, D., 1935. X-ray single-crystal photographs of insulin. Nature 135:591—592.

[52] Perutz, M.F., 1963. X-ray analysis of hemoglobin. Science 140(3569):863—869.

[53] Kendrew, J.C., 1963. Myoglobin and the structure of proteins. Science 139(3561):1259—1266.

[54] Blundell, T., Cutfield, J., Dodson, G., Dodson, E., Hodgkin, D., Mercola, D., 1971. The structure and biology of insulin. Biochemical Journal 125(3):50P—51P.

[55] Blundell, T., Dodson, G., Hodgkin, D., Mercola, D., 1972. Insulin: the structure in the crystal and its reflection in chemistry and biology by. Advances in Protein Chemistry 26:279—402.

[56] Chothia, C., Lesk, A.M., Dodson, G.G., Hodgkin, D.C., 1983. Transmission of conformational change in insulin. Nature 302(5908):500—505.

[57] Chothia, C., 1984. Principles that determine the structure of proteins. Annual Review of Biochemistry 53(1):537—572.

[58] Hodgkin, D.C., 1975. Chinese work on insulin. Nature 255(5504):103, 103.

[59] Blundell, T.L., Cutfield, J.F., Cutfield, S.M., Dodson, E.J., Dodson, G.G., Hodgkin, D.C., et al., 1971. Atomic positions in rhombohedral 2-zinc insulin crystals. Nature 231(5304):506—511.

[60] Chiti, F., Dobson, C.M., 2017. Protein misfolding, amyloid formation, and human disease: a summary of progress over the last decade. Annual Review of Biochemistry 86:27—68.

[61] Alexandrov, D.V., Nizovtseva, I.G., 2019. On the theory of crystal growth in metastable systems with biomedical applications: protein and insulin crystallization. Philosophical Transactions of the Royal Society A 377(2143):20180214.

[62] Jimenez, J.L., Nettleton, E.J., Bouchard, M., Robinson, C.V., Dobson, C.M., Saibil, H.R., 2002. The protofilament structure of insulin amyloid fibrils. Proceedings of the National Academy of Sciences 99(14):9196—9201.

[63] Balhorn, R., 2007. The protamine family of sperm nuclear proteins. Genome Biology 8(9):1—8.

[64] Hallas-Møller, K., Petersen, K., Schlichtkrull, J., 1952. Crystalline and amorphous insulin-zinc compounds with prolonged action. Science 116(3015):394—398.

[65] Hallas-Mø, K., 1956. The lente insulins. Diabetes 5(1):7—14.

[66] Wagner, A., Diez, J., Schulze-Briese, C., Schluckebier, G., 2009. Crystal structure of Ultralente—a microcrystalline insulin suspension. Proteins: Structure, Function, and Bioinformatics 74(4):1018—1027.

[67] Derewenda, U., Derewenda, Z., Dodson, E., Dodson, G., Reynolds, C., Smith, G., et al., 1989. Phenol stabilizes more helix in a new symmetrical zinc insulin hexamer. Nature 338(6216):594—596.

[68] Brader, M.L., Dunn, M.F., 1991. Insulin hexamers: new conformations and applications. Trends in Biochemical Sciences 16:341—345.

[69] Nanev, C.N., 2020. Advancements (and challenges) in the study of protein crystal nucleation and growth; thermodynamic and kinetic explanations and

comparison with small-molecule crystallization. Progress in Crystal Growth and Characterization of Materials 66(2):100484.

[70] Hagedorn, H.C., Jensen, B.N., Krarup, N., Wodstrup, I., 1936. Protamine insulinate. Journal of the American Medical Association 106(3):177—180.

[71] Hagedorn, H., 1937. Protamine insulinate. Proceedings of the Royal Society of Medicine 30:805—814.

[72] Miescher, F., 1874. Das Protamin, eine neue organische Base aus den Samenfäden des Rheinlachses. Berichte der Deutschen Chemischen Gesellschaft 7(1):376—379.

[73] Kossel, A., Pringle, H., 1906. Über Protamine und Histone. p. 301—21.

[74] Thompson, W., 1900. The physiological effects of protamines and their cleavage products. Transactions of the Royal Academy of Medicine in Ireland 18(1):426.

[75] Byrne, J., Miescher, D.R.F., 2019. The 150th anniversary of the discovery of DNA. Biomics.

[76] Hoffmann, J.A., Chance, R.E., Johnson, M.G., 1990. Purification and analysis of the major components of chum salmon protamine contained in insulin formulations using high-performance liquid chromatography. Protein Expression and Purification 1(2):127—133.

[77] Krayenbuhl, C., Rosenberg, T., 1946. Crystalline protamine insulin. Rep Steno Mem Hosp Nord Insulinlab 1:60—73.

[78] Fisher, A., 1955. Insulin preparations. Canadian Medical Association Journal 73(1):1.

[79] Deckert, T., Andersen, O.O., Pouken, J., 1974. The clinical significance of highly purified pig-insulin preparations. Diabetologia 10(6):703—708.

[80] Graham, D., Pomeroy, A., 1984. An in-vitro test for the duration of action of insulin suspensions. Journal of Pharmacy and Pharmacology 36(7):427—430.

[81] Kinnunen, H.M., Sharma, V., Contreras-Rojas, L.R., Yu, Y., Alleman, C., Sreedhara, A., et al., 2015. A novel in vitro method to model the fate of subcutaneously administered biopharmaceuticals and associated formulation components. Journal of Controlled Release 214:94—102.

[82] Bock, F., Lin, E., Larsen, C., Jensen, H., Huus, K., Larsen, S.W., et al., 2020. Towards in vitro in vivo correlation for modified release subcutaneously administered insulins. European Journal of Pharmaceutical Sciences 145: 105239.

[83] Heine, R., Bilo, H., Fonk, T., Van der Veen, E., Van der Meer, J., 1984. Absorption kinetics and action profiles of mixtures of short-and intermediate-acting insulins. Diabetologia 27(6):558—562.

[84] Woodworth, J.R., Howey, D.C., Bowsher, R.R., Brunelle, R.L., Rowe, H.M., Compton, J., et al., 1994. Comparative pharmacokinetics and glucodynamics of two human insulin mixtures 70/30 and 50/50 insulin mixtures. Diabetes Care 17(5):366—371.

[85] Balschmidt, P., Hansen, F.B., Dodson, E., Dodson, G., Korber, F., 1991. Structure of porcine insulin cocrystallized with clupeine Z. Acta Crystallographica Section B Structural Science 47(6):975—986.

[86] Kurtz, A., Gray, R., Markanday, S., Nabarro, J., 1983. Circulating IgG antibody to protamine in patients treated with protamine-insulins. Diabetologia 25(4): 322—324.

[87] Schernthaner, G., 1993. Immunogenicity and allergenic potential of animal and human insulins. Diabetes Care 16(Supplement 3):155—165.

[88] Markussen, J., Diers, I., Engesgaard, A., Hansen, M., Hougaard, P., Langkjaer, L., et al., 1987. Soluble, prolonged-acting insulin derivatives. II. Degree of protraction and crystallizability of insulins substituted in positions A17, B8, B13, B27 and B30. Protein Engineering Design and Selection 1(3): 215—223.

[89] Markussen, J., Diers, I., Hougaard, P., Langkjaer, L., Norris, K., Snel, L., et al., 1988. Soluble, prolonged-acting insulin derivatives. III. Degree of protraction, crystallizability and chemical stability of insulins substituted in positions A21, B13, B23, B27 and B30. Protein Engineering Design and Selection 2(2):157—166.

[90] Jørgensen, S., Vaag, A., Langkjær, L., Hougaard, P., Markussen, J., 1989. NovoSol Basal: pharmacokinetics of a novel soluble long acting insulin analogue. British Medical Journal 299(6696):415—419.

[91] Schade, D.S., Eaton, R.P., DeLongo, J., Saland, L.C., Ladman, A.J., Carlson, G.A., 1982. Electron microscopy of insulin precipitates. Diabetes Care 5(1):25—30.

[92] Levien, T.L., Baker, D.E., White Jr., J.R., Campbell, R.K., 2002. Insulin glargine: a new basal insulin. The Annals of Pharmacotherapy 36(6):1019—1027.

[93] Brown, M.A., Davis, C.S., Fleming, L.W., Fleming, J.W., 2016. The role of Toujeo®, insulin glargine U-300, in the treatment of diabetes mellitus. Journal of the American Association of Nurse Practitioners 28(9):503—509.

[94] Hilgenfeld, R., 1992. Controlling insulin bioavailability by crystal contact engineering. Diabetologia 35(1):A193.

[95] Pocock, S.J., Smeeth, L., 2009. Insulin glargine and malignancy: an unwarranted alarm. The Lancet 374(9689):511—513.

[96] Porcellati, F., Lucidi, P., Candeloro, P., Cioli, P., Andreoli, A.M., Curti, G., et al., 2019. 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 42(1):85—92.

[97] Whittingham, J.L., Havelund, S., Jonassen, I., 1997. Crystal structure of a prolonged-acting insulin with albumin-binding properties. Biochemistry 36(10):2826—2831.

[98] Ruiatkina, L., Sorokin, M.I., 2013. Detemir (Levemir): modern paradigms of insulin therapy. Problems of Endocrinology 59(4):56—64.

[99] Scherer, T., Sakamoto, K., Buettner, C., 2021. Brain insulin signalling in metabolic homeostasis and disease. Nature Reviews Endocrinology 17(8): 468—483.

[100] Steensgaard, D.B., Schluckebier, G., Strauss, H.M., Norrman, M., Thomsen, J.K., Friderichsen, A.V., et al., 2013. Ligand-controlled assembly of hexamers, dihexamers, and linear multihexamer structures by the engineered acylated insulin degludec. Biochemistry 52(2):295—309.

[101] Lau, J., Bloch, P., Schäffer, L., Pettersson, I., Spetzler, J., Kofoed, J., et al., 2015. Discovery of the once-weekly glucagon-like peptide-1 (GLP-1) analogue semaglutide. Journal of Medicinal Chemistry 58(18): 7370—7380.

[102] Cheang, J.Y., Moyle, P.M., 2018. Glucagon-like peptide-1 (GLP-1)-based therapeutics: current status and future opportunities beyond type 2 diabetes. ChemMedChem 13(7):662—671.

[103] Nauck, M.A., Wefers, J., Meier, J.J., 2021. Treatment of type 2 diabetes: challenges, hopes, and anticipated successes. Lancet Diabetes Endocrinology 9(8):525—544.

[104] Kjeldsen, T.B., Hubálek, F.e., Hjørringgaard, C.U., Tagmose, T.M., Nishimura, E., Stidsen, C.E., et al., 2021. Molecular engineering of insulin Icodec, the first acylated insulin analog for once-weekly administration in humans. Journal of Medicinal Chemistry 64:8942—8950.

[105] Rosenstock, J., Bajaj, H.S., Janež, A., Silver, R., Begtrup, K., Hansen, M.V., et al., 2020. Once-weekly insulin for type 2 diabetes without previous insulin treatment. New England Journal of Medicine 383(22):2107—2116.

[106] Bajaj, H.S., Bergenstal, R.M., Christoffersen, A., Davies, M.J., Gowda, A., Isendahl, J., et al., 2021. Switching to once-weekly insulin Icodec versus once-daily insulin glargine U100 in type 2 diabetes inadequately controlled on daily basal insulin: a phase 2 randomized controlled trial. Diabetes Care dc202877, doi: 202810.202337/dc202820-202877.

[107] Nakagawa, S.H., Tager, H., 1986. Role of the phenylalanine B25 side chain in directing insulin interaction with its receptor. Steric and conformational effects. Journal of Biological Chemistry 261(16):7332—7341.

[108] Mirmira, R., Nakagawa, S.H., Tager, H., 1991. Importance of the character and configuration of residues B24, B25, and B26 in insulin-receptor interactions. Journal of Biological Chemistry 266(3):1428—1436.

[109] Ludvigsen, S., Roy, M., Thoegersen, H., Kaarsholm, N.C., 1994. High-resolution structure of an engineered biologically potent insulin monomer B16 Tyr→His is, as determined by nuclear magnetic resonance spectroscopy. Biochemistry 33(26):7998—8006.

[110] Ludvigsen, S., Olsen, H.B., Kaarsholm, N.C., 1998. A structural switch in a mutant insulin exposes key residues for receptor binding. Journal of Molecular Biology 279(1):1—7.

[111] Kjeldsen, T.B., Hubálek, F.e., Tagmose, T.M., Pridal, L., Refsgaard, H.H., Porsgaard, T., et al., 2020. Engineering of orally available, ultralong-acting insulin analogues: discovery of OI338 and OI320. Journal of Medicinal Chemistry 64(1):616—628.

[112] Rath, T., Baker, K., Dumont, J.A., Peters, R.T., Jiang, H., Qiao, S.-W., et al., 2015. Fc-fusion proteins and FcRn: structural insights for longer-lasting and more effective therapeutics. Critical Reviews in Biotechnology 35(2):235—254.

[113] Baghban Taraghdari, Z., Imani, R., Mohabatpour, F., 2019. A review on bioengineering approaches to insulin delivery: a pharmaceutical and engineering perspective. Macromolecular Bioscience 19(4):1800458.

[114] Moore, M.C., Smith, M.S., Sinha, V.P., Beals, J.M., Michael, M.D., Jacober, S.J., et al., 2014. Novel PEGylated basal insulin LY2605541 has a preferential hepatic effect on glucose metabolism. Diabetes 63(2):494—504.

[115] Sinha, V., Howey, D., Choi, S., Mace, K., Heise, T., 2014. Steady-state pharmacokinetics and glucodynamics of the novel, long-acting basal insulin LY2605541 dosed once-daily in patients with type 2 diabetes mellitus. Diabetes, Obesity and Metabolism 16(4):344—350.

[116] Hedrington, M.S., Davis, S.N., 2017. Discontinued drug therapies to treat diabetes in 2015. Expert Opinion on Investigational Drugs 26(2):219—225.

[117] Carter, P.J., 2011. Introduction to current and future protein therapeutics: a protein engineering perspective. Experimental Cell Research 317(9):1261—1269.

[118] Jafari, R., M Zolbanin, N., Rafatpanah, H., Majidi, J., Kazemi, T., 2017. Fc-fusion proteins in therapy: an updated view. Current Medicinal Chemistry 24(12):1228—1237.

[119] Kuo, T.T., Aveson, V.G., 2011. Neonatal F_c receptor and IgG-based therapeutics. MAbs: Taylor & Francis. p. 422—30.

[120] Heise, T., Chien, J., Beals, J., Benson, C., Klein, O., Moyers, J.S., et al., 2021. Basal Insulin FC (BIF), a novel insulin suited for once weekly dosing for the treatment of patients with diabetes mellitus. Journal of the Endocrine Society 5(Supplement 1):A329. A329.

[121] Heise, T., 2021. The future of insulin therapy. Diabetes Research and Clinical Practice 175:108820.

[122] Wronkowitz, N., Hartmann, T., Görgens, S.W., Dietze-Schroeder, D., Indrakusuma, I., Choi, I.Y., et al., 2017. LAPSInsulin115: a novel ultra-long-acting basal insulin with a unique action profile. Diabetes, Obesity and Metabolism 19(12):1722—1731.

[123] Faust, C., Ochs, C., Korn, M., Werner, U., Jung, J., Dittrich, W., et al., 2020. Production of a novel heterodimeric two-chain insulin-Fc fusion protein. Protein Engineering Design and Selection 33. https://doi.org/10.1093/protein/gzaa1026.

[124] Cherrington, A.D., Sindelar, D., Edgerton, D., Steiner, K., McGuinness, O.P., 2002. Physiological consequences of phasic insulin release in the normal animal. Diabetes 51(suppl 1):S103—S108.

[125] Del Prato, S., Marchetti, P., Bonadonna, R.C., 2002. Phasic insulin release and metabolic regulation in type 2 diabetes. Diabetes 51(Supplement 1): S109—S116.

[126] Sherwin, R.S., Kramer, K.J., Tobin, J.D., Insel, P.A., Liljenquist, J.E., Berman, M., et al., 1974. A model of the kinetics of insulin in man. Journal of Clinical Investigation 53(5):1481—1492.

[127] Joseph, J.I., Torjman, M.C., 1999. The Artificial Pancreas Symposium: glucose monitoring, insulin delivery, feedback control. Diabetes Technology & Therapeutics 1(3):323—336.

[128] He, J., Renard, E., Lord, P., Cohen, D., Cem-Duranty, E., Place, J., et al., 2021. Root cause determination of intraperitoneal catheter obstructions: insulin amyloid aggregates vs foreign body reaction. Journal of Controlled Release 336:1—15.

[129] Heinemann, L., Traut, T., Heise, T., 1997. Time—action profile of inhaled insulin. Diabetic Medicine 14(1):63—72.

[130] Cavaiola, T.S., Edelman, S., 2014. Inhaled insulin: a breath of fresh air? A review of inhaled insulin. Clinical Therapeutics 36(8):1275—1289.

[131] Bode, B.W., McGill, J.B., Lorber, D.L., Gross, J.L., Chang, P.-C., Bregman, D.B., 2015. Inhaled technosphere insulin compared with injected prandial insulin in type 1 diabetes: a randomized 24-week trial. Diabetes Care 38(12):2266—2273.

[132] Levin, P., Hoogwerf, B.J., Snell-Bergeon, J., Vigers, T., Pyle, L., Bromberger, L., 2021. Ultra rapid-acting inhaled insulin improves glucose control in patients with type 2 diabetes mellitus. Endocrine Practice 27(5): 449—454.

[133] Spaan, N., Teplova, A., Stam, G., Spaan, J., Lucas, C., 2014. Systematic review: continuous intraperitoneal insulin infusion with implantable insulin pumps for diabetes mellitus. Acta Diabetologica 51(3):339—351.

[134] Dirnena-Fusini, I., Åm, M.K., Fougner, A.L., Carlsen, S.M., Christiansen, S.C., 2021. Physiological effects of intraperitoneal versus subcutaneous insulin infusion in patients with diabetes mellitus type 1: a systematic review and meta-analysis. PloS One 16(4):e0249611.

[135] Hovorka, R., 2006. Continuous glucose monitoring and closed-loop systems. Diabetic Medicine 23(1):1—12.

[136] Renard, E., Place, J., Cantwell, M., Chevassus, H., Palerm, C.C., 2010. Closed-loop insulin delivery using a subcutaneous glucose sensor and intraperitoneal insulin delivery: feasibility study testing a new model for the artificial pancreas. Diabetes Care 33(1):121—127.

[137] Doyle, F.J., Huyett, L.M., Lee, J.B., Zisser, H.C., Dassau, E., 2014. Closed-loop artificial pancreas systems: engineering the algorithms. Diabetes Care 37(5):1191—1197.

[138] Boughton, C.K., Hovorka, R., 2021. New closed-loop insulin systems. Diabetologia, 1—9.

[139] Goeddel, D.V., Kleid, D.G., Bolivar, F., Heyneker, H.L., Yansura, D.G., Crea, R., et al., 1979. Expression in Escherichia coli of chemically synthesized genes for human insulin. Proceedings of the National Academy of Sciences 76(1): 106—110.

[140] Johnson, I.S., 1983. Human insulin from recombinant DNA technology. Science 219(4585):632—637.

[141] Brange, J., Ribel, U., Hansen, J., Dodson, G., Hansen, M., Havelund, S., et al., 1988. Monomeric insulins obtained by protein engineering and their medical implications. Nature 333(6174):679—682.

[142] Hansen, B., Kurtzhals, P., Jensen, A.B., Dejgaard, A., Russell-Jones, D., 2011. Insulin X10 revisited: a super-mitogenic insulin analogue. Diabetologia 54(9):2226—2231.

[143] Hansen, B.F., Glendorf, T., Hegelund, A.C., Lundby, A., Lützen, A., Slaaby, R., et al., 2012. Molecular characterisation of long-acting insulin analogues in comparison with human insulin, IGF-1 and insulin X10. PloS One 7(5): e34274.

[144] Chan, S.J., Seino, S., Gruppuso, P.A., Schwartz, R., Steiner, D.F., 1987. A mutation in the B chain coding region is associated with impaired proinsulin conversion in a family with hyperproinsulinemia. Proceedings of the National Academy of Sciences 84(8):2194—2197.

[145] Carroll, R.J., Hammer, R.E., Chan, S.J., Swift, H.H., Rubenstein, A.H., Steiner, D.F., 1988. A mutant human proinsulin is secreted from islets of Langerhans in increased amounts via an unregulated pathway. Proceedings of the National Academy of Sciences 85(23):8943—8947.

[146] Schwartz, G.P., Burke, G.T., Katsoyannis, P.G., 1987. A superactive insulin: [B10-aspartic acid] insulin (human). Proceedings of the National Academy of Sciences 84(18):6408—6411.

[147] Brems, D.N., Alter, L.A., Beckage, M.J., Chance, R.E., DiMarchi, R.D., Green, L.K., et al., 1992. Altering the association properties of insulin by amino acid replacement. Protein Engineering Design and Selection 5(6): 527—533.

[148] Jeitler, K., Horvath, K., Berghold, A., Gratzer, T., Neeser, K., Pieber, T., et al., 2008. Continuous subcutaneous insulin infusion versus multiple daily insulin injections in patients with diabetes mellitus: systematic review and meta-analysis. Diabetologia 51(6):941—951.

[149] Freckmann, G., Buck, S., Waldenmaier, D., Kulzer, B., Schnell, O., Gelchsheimer, U., et al., 2020. Insulin pump therapy for patients with type 2 diabetes mellitus: evidence, current barriers, and new technologies. Journal of Diabetes Science and Technology 15(4):901—915.

[150] Boscari, F., Avogaro, A., 2021. Current treatment options and challenges in patients with Type 1 diabetes: pharmacological, technical advances and future perspectives. Reviews in Endocrine & Metabolic Disorders 22(2):217—240.

[151] Woods, R.J., Alarcón, J., McVey, E., Pettis, R.J., 2012. Intrinsic fibrillation of fast-acting insulin analogs. Journal of Diabetes Science and Technology 6(2): 265—276.

[152] Krasner, A., Pohl, R., Simms, P., Pichotta, P., Hauser, R., De Souza, E., 2012. A review of a family of ultra-rapid-acting insulins: formulation development. Journal of Diabetes Science and Technology 6(4):786—796.

[153] Owens, D.R., Bolli, G.B., 2020. The continuing quest for better subcutaneously administered prandial insulins: a review of recent developments and potential clinical implications. Diabetes, Obesity and Metabolism 22(5):743—754.

[154] Falk, B.T., Liang, Y., McCoy, M.A., 2020. Profiling insulin oligomeric states by 1H NMR spectroscopy for formulation development of ultra-rapid-acting insulin. Journal of Pharmaceutical Sciences 109(1):922—926.

[155] Boughton, C.K., Hartnell, S., Thabit, H., Poettler, T., Herzig, D., Wilinska, M.E., et al., 2021. Hybrid closed-loop glucose control with faster insulin aspart compared with standard insulin aspart in adults with type 1 diabetes: a double-blind, multicentre, multinational, randomized, crossover study. Diabetes, Obesity and Metabolism 23(6):1389—1396.

[156] Ciszak, E., Beals, J.M., Frank, B.H., Baker, J.C., Carter, N.D., Smith, G.D., 1995. Role of C-terminal B-chain residues in insulin assembly: the structure of hexameric Lys^{B28}, Pro^{B29}-human insulin. Structure 3(6):615—622.

[157] Whittingham, J.L., Edwards, D.J., Antson, A.A., Clarkson, J.M., Dodson, G.G., 1998. Interactions of phenol and m-cresol in the insulin hexamer, and their effect on the association properties of B28 Pro→Asp insulin analogues. Biochemistry 37(33):11516—11523.

[158] Hua, Q.X., Shoelson, S.E., Kochoyan, M., Weiss, M.A., 1991. Receptor binding redefined by a structural switch in a mutant human insulin. Nature 354(6350):238—241.

[159] Hua, Q.-X., Hu, S.-Q., Frank, B.H., Jia, W., Chu, Y.-C., Wang, S.-H., et al., 1996. Mapping the functional surface of insulin by design: structure and function of a novel A-chain analogue. Journal of Molecular Biology 264(2): 390—403.

[160] Bentley, G., Dodson, E., Dodson, G., Hodgkin, D., Mercola, D., 1976. Structure of insulin in 4-zinc insulin. Nature 261(5556):166—168.

[161] Gillis, R.B., Solomon, H.V., Govada, L., Oldham, N.J., Dinu, V., Jiwani, S.I., et al., 2021. Analysis of insulin glulisine at the molecular level by X-ray crystallography and biophysical techniques. Scientific Reports 11(1):1—10.

[162] Warren, M., Bode, B., Cho, J.I., Liu, R., Tobian, J., Hardy, T., et al., 2021. Improved postprandial glucose control with ultra-rapid lispro versus lispro with continuous subcutaneous insulin infusion in type 1 diabetes: PRONTO-Pump-2. Diabetes, Obesity and Metabolism 23(7):1552—1561.

[163] Berenson, D.F., Weiss, A.R., Wan, Z.-l., Weiss, M.A., 2011. Insulin analogs for the treatment of diabetes mellitus: therapeutic applications of protein engineering. Annals of the New York Academy of Sciences 1243:E40—E54.

[164] Haahr, H., Heise, T., 2020. Fast-acting insulin aspart: a review of its pharmacokinetic and pharmacodynamic properties and the clinical consequences. Clinical Pharmacokinetics 59(2):155—172.

[165] Pratt, E., Leohr, J., Heilmann, C., Johnson, J., Landschulz, W., 2017. Treprostinil causes local vasodilation, is well tolerated, and results in faster absorption of insulin lispro, (Abstract) American Diabetes Association, 1701 N. VA 22311-1717 USA: Beauregard St., Alexandra. p. A253. A253.

[166] Paavola, C.D., Cox, A.L., Sperry, A.E., Hansen, R.J., Li, S., Bradley, S.A., et al., 2017. A stable, hexameric, ultra-rapid insulin formulation containing citrate (Abstract). VA 22311-1717 USA: American Diabetes Association, 1701 N. Beauregard St., Alexandra. p. A254. A254.

[167] Blevins, T., Zhang, Q., Frias, J.P., Jinnouchi, H., Chang, A.M., 2020. Randomized double-blind clinical trial comparing Ultra rapid lispro with Lispro in a basal-bolus regimen in patients with type 2 diabetes: PRONTO-T2D. Diabetes Care 43(12):2991—2998.

[168] Linnebjerg, H., Zhang, Q., LaBell, E., Dellva, M.A., Coutant, D.E., Hövelmann, U., et al., 2020. Pharmacokinetics and glucodynamics of ultra rapid lispro (URLi) versus Humalog®($lispro$) in younger adults and elderly patients with type 1 diabetes mellitus: a randomised controlled trial. Clinical Pharmacokinetics 59:1589—1599.

[169] Meiffren, G., Plum-Moerschel, L., Ranson, A., Anastassiadis, E., Seroussi, C., Correia, J., et al., 2018. BioChaperone 222 (BC222), the new excipient enabling the ultra-rapid BioChaperone lispro (BCLIS) formulation, is completely absorbed and rapidly excreted after subcutaneous (sc) injection. American Diabetes Association. https://doi.org/10.2337/db2318-1024-.

[170] Heise, T., Meiffren, G., Alluis, B., Seroussi, C., Ranson, A., Arrubla, J., et al., 2019. BioChaperone Lispro versus faster aspart and insulin aspart in patients with type 1 diabetes using continuous subcutaneous insulin infusion: a randomized euglycemic clamp study. Diabetes, Obesity and Metabolism 21(4):1066—1070.

[171] Mann, J.L., Maikawa, C.L., Smith, A.A., Grosskopf, A.K., Baker, S.W., Roth, G.A., et al., 2020. An ultrafast insulin formulation enabled by high-throughput screening of engineered polymeric excipients. Science Translational Medicine 12(550):eaba6676.

[172] Maikawa, C.L., d'Aquino, A.I., Lal, R.A., Buckingham, B.A., Appel, E.A., 2021. Engineering biopharmaceutical formulations to improve diabetes management. Science Translational Medicine 13(578):eabd6726.

[173] Bakh, N.A., Cortinas, A.B., Weiss, M.A., Langer, R.S., Anderson, D.G., Gu, Z., et al., 2017. Glucose-responsive insulin by molecular and physical design. Nature Chemistry 9(10):937.

[174] VandenBerg, M.A., Webber, M.J., 2019. Biologically inspired and chemically derived methods for glucose-responsive insulin therapy. Advanced Healthcare Materials 8(12):1801466.

[175] Hoeg-Jensen, T., 2021. Glucose-sensitive insulin. Molecular Metabolism 46: 101—107.

[176] James, T.D., Sandanayake, K.S., Shinkai, S., 1996. Saccharide sensing with molecular receptors based on boronic acid. Angewandte Chemie International Edition 35(17):1910—1922.

[177] Springsteen, G., Wang, B., 2002. A detailed examination of boronic acid—diol complexation. Tetrahedron 58(26):5291—5300.

[178] Hoeg-Jensen, T., Ridderberg, S., Havelund, S., Schäffer, L., Balschmidt, P., Jonassen, I., et al., 2005. Insulins with built-in glucose sensors for glucose responsive insulin release. Journal of Peptide Science 11(6):339—346.

[179] Chou, D.H.-C., Webber, M.J., Tang, B.C., Lin, A.B., Thapa, L.S., Deng, D., et al., 2015. Glucose-responsive insulin activity by covalent modification with aliphatic phenylboronic acid conjugates. Proceedings of the National Academy of Sciences 112(8):2401—2406.

[180] Kruse, T., Kofoed-Hansen, M., Muenzel, M.W.B., Thoegersen, H., Sauerberg, P., Rasmussen, J.E., et al., 2020. Glucose-sensitive albumin-binding derivatives. WO Patent Application WO/2019/092125. p. 2028. May 16, 2019; U.S. Patent Application no. 16759378, August 11.

[181] Peters, J.A., 2014. Interactions between boric acid derivatives and saccharides in aqueous media: structures and stabilities of resulting esters. Coordination Chemistry Reviews 268:1—22.

[182] Bian, Z., Liu, A., Li, Y., Fang, G., Yao, Q., Zhang, G., et al., 2020. Boronic acid sensors with double recognition sites: a review. Analyst 145(3):719—744.

[183] Shen, D., Yu, H., Wang, L., Khan, A., Haq, F., Chen, X., et al., 2020. Recent progress in design and preparation of glucose-responsive insulin delivery systems. Journal of Controlled Release 321:236—258.

[184] Wang, Y., Huang, F., Sun, Y., Gao, M., Chai, Z., 2017. Development of shell cross-linked nanoparticles based on boronic acid-related reactions for self-regulated insulin delivery. Journal of Biomaterials Science, Polymer Edition 28(1):93—106.

[185] Wang, J., Yu, J., Zhang, Y., Kahkoska, A.R., Wang, Z., Fang, J., et al., 2019. Glucose transporter inhibitor-conjugated insulin mitigates hypoglycemia. Proceedings of the National Academy of Sciences 116(22):10744—10748.

[186] Wang, J., Wang, Z., Yu, J., Zhang, Y., Zeng, Y., Gu, Z., 2019. A forskolin-conjugated insulin analog targeting endogenous glucose-transporter for glucose-responsive insulin delivery. Biomaterials science 7(11):4508—4513.

[187] Kaarsholm, N.C., Lin, S., Yan, L., Kelly, T., van Heek, M., Mu, J., et al., 2018. Engineering glucose responsiveness into insulin. Diabetes 67(2):299—308.

[188] Chen, Y.-S., Gleaton, J., Yang, Y., Dhayalan, B., Phillips, N.B., Liu, Y., et al., 2021. Insertion of a synthetic switch into insulin provides metabolite-dependent regulation of hormone-receptor activation. Proceedings of the National Academy of Sciences 118(30). https://doi.org/10.1073/pnas.2103518118.

[189] Menting, J.G., Yang, Y., Chan, S.J., Phillips, N.B., Smith, B.J., Whittaker, J., et al., 2014. Protective hinge in insulin opens to enable its receptor engagement. Proceedings of the National Academy of Sciences 111(33):E3395—E3404.

[190] Steiner, K.E., Williams, P.E., Lacy, W.W., Cherrington, A.D., 1990. Effects of insulin on glucagon-stimulated glucose production in the conscious dog. Metabolism 39(12):1325—1333.

[191] Dobbins, R.L., Connolly, C.C., Neal, D.W., Palladino, L.J., Parlow, A.F., Cherrington, A.D., 1991. Role of glucagon in countering hypoglycemia induced by insulin infusion in dogs. American Journal of Physiology - Endocrinology And Metabolism 261(6):E773—E781.

[192] Rivera, N., Ramnanan, C.J., An, Z., Farmer, T., Smith, M., Farmer, B., et al., 2010. Insulin-induced hypoglycemia increases hepatic sensitivity to glucagon in dogs. Journal of Clinical Investigation 120(12):4425—4435.

[193] Bode, B.W., Boyd, J., Shah, A., Parkes, D., Ghosh, S., Cherrington, A.D., 2020. 7-LB: insulin and glucagon coadministration in type 1 diabetes prevents hypoglycemia without worsening hyperglycemia. Diabetes pp. doi: 0.2337/db2320-2337-LB.

[194] Pedersen, C., Bouman, S.D., Porsgaard, T., Rosenkilde, M.M., Roed, N.K., 2018. Dual treatment with a fixed ratio of glucagon and insulin increases the therapeutic window of insulin in diabetic rats. Physiological reports 6(6):e13657.

[195] Pedersen, C., Porsgaard, T., Thomsen, M., Rosenkilde, M.M., Roed, N.K., 2018. Sustained effect of glucagon on body weight and blood glucose: assessed by continuous glucose monitoring in diabetic rats. PloS One 13(3):e0194468.

[196] Yang, Q., Vijayakumar, A., Kahn, B.B., 2018. Metabolites as regulators of insulin sensitivity and metabolism. Nature Reviews Molecular Cell Biology 19(10):654—672.

[197] Kahn, B.B., 2019. Adipose tissue, inter-organ communication, and the path to type 2 diabetes: the 2016 banting medal for scientific achievement lecture. Diabetes 68(1):3—14.

[198] Santoro, A., McGraw, T.E., Kahn, B.B., 2021. Insulin action in adipocytes, adipose remodeling, and systemic effects. Cell Metabolism 33(4):748—757.

[199] Shao, J., Zaro, J.L., Shen, W.-C., 2016. Tissue barriers and novel approaches to achieve hepatoselectivity of subcutaneously-injected insulin therapeutics. Tissue Barriers 4(2):e1156804.

[200] Caparrotta, T., Evans, M., 2014. PEGylated insulin Lispro,(LY2605541)—a new basal insulin analogue. Diabetes, Obesity and Metabolism 16(5):388—395.

[201] Areias, M.F.C., Prada, P.O., 2015. Mechanisms of insulin resistance in the amygdala: influences on food intake. Behavioural Brain Research 282:209—217.

[202] Mitchell, C.S., Begg, D.P., 2021. The regulation of food intake by insulin in the central nervous system. Journal of Neuroendocrinology 33(4):e12952.

[203] Grattan, D.R., Andrews, Z.B., 2021. Insulin as a neuroendocrine hormone. Journal of Neuroendocrinology 33(4):e12966.

[204] Belsham, D.D., Dalvi, P.S., 2021. Insulin signalling in hypothalamic neurones. Journal of Neuroendocrinology 33(4):e12919.

[205] Petrovich, G.D., Setlow, B., Holland, P.C., Gallagher, M., 2002. Amygdalo-hypothalamic circuit allows learned cues to override satiety and promote eating. Journal of Neuroscience 22(19):8748—8753.

[206] Hallschmid, M., Jauch-Chara, K., Korn, O., Mölle, M., Rasch, B., Born, J., et al., 2010. Euglycemic infusion of insulin detemir compared with human insulin appears to increase direct current brain potential response and reduces food intake while inducing similar systemic effects. Diabetes 59(4):1101—1107.

[207] Tsuneki, H., Yoshida, H., Endo, K., Mori, N., Hosoh, S., Tsuda, M., et al., 2017. Different impacts of acylated and non-acylated long-acting insulin analogs on neural functions in vitro and in vivo. Diabetes Research and Clinical Practice 129:62—72.

[208] Hölscher, C., 2020. Brain insulin resistance: role in neurodegenerative disease and potential for targeting. Expert Opinion on Investigational Drugs 29(4):333—348.

[209] Heidenreich, K.A., Zahniser, N.R., Berhanu, P., Brandenburg, D., Olefsky, J.M., 1983. Structural differences between insulin receptors in the brain and peripheral target tissues. Journal of Biological Chemistry 258(14):8527—8530.

[210] Ferreira, S.T., 2021. Brain insulin, insulin-like growth factor 1 and glucagon-like peptide 1 signalling in Alzheimer's disease. Journal of Neuroendocrinology 33(4):e12959.

[211] Choi, C.H.J., Cohen, P., 2017. Adipose crosstalk with other cell types in health and disease. Experimental Cell Research 360(1):6—11.

[212] Cao, H., Gerhold, K., Mayers, J.R., Wiest, M.M., Watkins, S.M., Hotamisligil, G.S., 2008. Identification of a lipokine, a lipid hormone linking adipose tissue to systemic metabolism. Cell 134(6):933—944.

[213] Yore, M.M., Syed, I., Moraes-Vieira, P.M., Zhang, T., Herman, M.A., Homan, E.A., et al., 2014. Discovery of a class of endogenous mammalian lipids with anti-diabetic and anti-inflammatory effects. Cell 159(2):318—332.

[214] Turpin, S.M., Nicholls, H.T., Willmes, D.M., Mourier, A., Brodesser, S., Wunderlich, C.M., et al., 2014. Obesity-induced CerS6-dependent C16:0 ceramide production promotes weight gain and glucose intolerance. Cell Metabolism 20(4):678—686.

[215] Raichur, S., Brunner, B., Bielohuby, M., Hansen, G., Pfenninger, A., Wang, B., et al., 2019. The role of C16:0 ceramide in the development of obesity and type 2 diabetes: CerS6 inhibition as a novel therapeutic approach. Molecular Metabolism 21:36—50.

[216] Congreve, M., de Graaf, C., Swain, N.A., Tate, C.G., 2020. Impact of GPCR structures on drug discovery. Cell 181(1):81—91.

[217] Whalen, E.J., Rajagopal, S., Lefkowitz, R.J., 2011. Therapeutic potential of β-arrestin-and G protein-biased agonists. Trends in Molecular Medicine 17(3):126—139.

[218] Rajagopal, S., Rajagopal, K., Lefkowitz, R.J., 2010. Teaching old receptors new tricks: biasing seven-transmembrane receptors. Nature Reviews Drug Discovery 9(5):373—386.

[219] Machelska, H., Celik, M.Ö., 2020. Opioid receptors in immune and glial cells—implications for pain control. Frontiers in Immunology 11:300.

[220] Smith, J.S., Nicholson, L.T., Suwanpradid, J., Glenn, R.A., Knape, N.M., Alagesan, P., et al., 2018. Biased agonists of the chemokine receptor CXCR3 differentially control chemotaxis and inflammation. Science Signaling 11(555).

[221] Brown, M.S., Goldstein, J.L., 2008. Selective versus total insulin resistance: a pathogenic paradox. Cell Metabolism 7(2):95—96.

[222] Rodríguez-Flores, M., García-García, E., Cano-Nigenda, C.V., Cantú-Brito, C., 2014. Relationship of obesity and insulin resistance with the cerebrovascular reactivity: a case control study. Cardiovascular Diabetology 13(1):1—7.

[223] Sekizkardes, H., Chung, S.T., Chacko, S., Haymond, M.W., Startzell, M., Walter, M., et al., 2020. Free fatty acid processing diverges in human pathologic insulin resistance conditions. Journal of Clinical Investigation 130(7):3592—3602.

[224] Saltiel, A.R., Kahn, C.R., 2001. Insulin signalling and the regulation of glucose and lipid metabolism. Nature 414(6865):799—806.

[225] Rahman, I., Athar, M.T., Islam, M., 2020. Type 2 diabetes, obesity, and cancer share some common and critical pathways. Frontiers in Oncology 10: 600824.

[226] Krüger, M., Kratchmarova, I., Blagoev, B., Tseng, Y.-H., Kahn, C.R., Mann, M., 2008. Dissection of the insulin signaling pathway via quantitative phosphoproteomics. Proceedings of the National Academy of Sciences 105(7):2451—2456.

[227] Kalita, B., Bano, S., Vavachan, V.M., Taunk, K., Seshadri, V., Rapole, S., 2020. Application of mass spectrometry based proteomics to understand diabetes: a special focus on interactomics. Biochimica et Biophysica Acta (BBA) - Proteins & Proteomics 1868(10):140469.

[228] Pillutla, R.C., Hsiao, K.-c., Beasley, J.R., Brandt, J., Østergaard, S., Hansen, P.H., et al., 2002. Peptides identify the critical hotspots involved in the biological activation of the insulin receptor. Journal of Biological Chemistry 277(25):22590—22594.

[229] Jensen, M., Palsgaard, J., Borup, R., De Meyts, P., Schäffer, L., 2008. Activation of the insulin receptor (IR) by insulin and a synthetic peptide has different effects on gene expression in IR-transfected L6 myoblasts. Biochemical Journal 412(3):435—445.

[230] Lawrence, C.F., Margetts, M.B., Menting, J.G., Smith, N.A., Smith, B.J., Ward, C.W., et al., 2016. Insulin mimetic peptide disrupts the primary binding site of the insulin receptor. Journal of Biological Chemistry 291(30):15473—15481.

[231] Bacon, K., Blain, A., Burroughs, M., McArthur, N., Rao, B.M., Menegatti, S., 2020. Isolation of chemically cyclized peptide binders using yeast surface display. ACS Combinatorial Science 22(10):519—532.

[232] Iskandar, S.E., Haberman, V.A., Bowers, A.A., 2020. Expanding the chemical diversity of genetically encoded libraries. ACS Combinatorial Science 22(12): 712—733.

[233] Bowen, J., Schneible, J., Bacon, K., Labar, C., Menegatti, S., Rao, B.M., 2021. Screening of yeast display libraries of enzymatically treated peptides to discover macrocyclic peptide ligands. International Journal of Molecular Sciences 22(4):1634.

[234] Jeong, M.-Y., Rutter, J., Chou, D.H.-C., 2018. Display of single-chain insulin-like peptides on a yeast surface. Biochemistry 58(3):182—188.

[235] Savage, D.B., Semple, R.K., Chatterjee, V.K.K., Wales, J.K., Ross, R.J., O'Rahilly, S., 2007. A clinical approach to severe insulin resistance. Congenital Endocrinopathies 11:122—132.

[236] Schloot, N.C., Hood, R.C., Corrigan, S.M., Panek, R.L., Heise, T., 2019. Concentrated insulins in current clinical practice. Diabetes Research and Clinical Practice 148:93—101.

[237] Sun, G., Kashyap, S.R., 2011. Cancer risk in type 2 diabetes mellitus: metabolic links and therapeutic considerations. Journal of Nutrition and Metabolism, 708183.

[238] Kurtzhals, P., Schäffer, L., Sørensen, A., Kristensen, C., Jonassen, I., Schmid, C., et al., 2000. Correlations of receptor binding and metabolic and mitogenic potencies of insulin analogs designed for clinical use. Diabetes 49(6):999—1005.

[239] Currie, C.J., Poole, C.D., Gale, E., 2009. The influence of glucose-lowering therapies on cancer risk in type 2 diabetes. Diabetologia 52(9):1766—1777.

[240] But, A., De Bruin, M.L., Bazelier, M.T., Hjellvik, V., Andersen, M., Auvinen, A., et al., 2017. Cancer risk among insulin users: comparing analogues with human insulin in the CARING five-country cohort study. Diabetologia 60(9): 1691—1703.

[241] Versteyhe, S., Klaproth, B., Borup, R., Palsgaard, J., Jensen, M., Gray, S.G., et al., 2013. IGF-I, IGF-II, and insulin stimulate different gene expression responses through binding to the IGF-I receptor. Frontiers in Endocrinology 4: 98.

[242] Moreira, R.O., Cobas, R., Coelho, R.C.L.A., 2018. Combination of basal insulin and GLP-1 receptor agonist: is this the end of basal insulin alone in the treatment of type 2 diabetes? Diabetology & Metabolic Syndrome 10(1):1—10.

[243] Rodbard, H.W., Lingvay, I., Reed, J., De La Rosa, R., Rose, L., Sugimoto, D., et al., 2018. Semaglutide added to basal insulin in type 2 diabetes (SUSTAIN 5): a randomized, controlled trial. Journal of Clinical Endocrinology & Metabolism 103(6):2291—2301.

[244] Perreault, L., Rodbard, H., Valentine, V., Johnson, E., 2019. Optimizing fixed-ratio combination therapy in type 2 diabetes. Advances in Therapy 36(2): 265—277.

[245] Nuffer, W., Guesnier, A., Trujillo, J.M., 2018. A review of the new GLP-1 receptor agonist/basal insulin fixed-ratio combination products. Therapeutic Advances in Endocrinology and Metabolism 9(3):69—79.

[246] Castellana, M., Cignarelli, A., Brescia, F., Laviola, L., Giorgino, F., 2019. GLP-1 receptor agonist added to insulin versus basal-plus or basal-bolus insulin therapy in type 2 diabetes: a systematic review and meta-analysis. Diabetes 35(1):e3082.

[247] Huthmacher, J.A., Meier, J.J., Nauck, M.A., 2020. Efficacy and safety of short-and long-acting glucagon-like peptide 1 receptor agonists on a background of basal insulin in type 2 diabetes: a meta-analysis. Diabetes Care 43(9):2303—2312.

[248] Riddle, M.C., Nahra, R., Han, J., Castle, J., Hanavan, K., Hompesch, M., et al., 2018. Control of postprandial hyperglycemia in type 1 diabetes by 24-hour fixed-dose coadministration of pramlintide and regular human insulin: a randomized, two-way crossover study. Diabetes Care 41(11):2346—2352.

[249] Riddle, M.C., 2020. Rediscovery of the second β-cell hormone: Co-replacement with pramlintide and insulin in type 1 diabetes. Diabetes Care 43(3):518—521.

[250] Haidar, A., Tsoukas, M.A., Bernier-Twardy, S., Yale, J.-F., Rutkowski, J., Bossy, A., et al., 2020. A novel dual-hormone insulin-and-pramlintide artificial pancreas for type 1 diabetes: a randomized controlled crossover trial. Diabetes Care 43(3):597—606.

[251] Frias, J.P., Chien, J., Zhang, Q., Chigutsa, E., Landschulz, W., Wullenweber, P., et al., 2021. Once Weekly Basal Insulin F_c (BIF) is safe and efficacious in patients with type 2 diabetes mellitus (T2DM) previously treated with basal insulin. Journal of the Endocrine Society 5(Supplement 1):A448—A449.

[252] Gonzalez, J.S., Tanenbaum, M.L., Commissariat, P.V., 2016. Psychosocial factors in medication adherence and diabetes self-management: implications for research and practice. American Psychologist 71(7):539.

[253] Weiss, M.A., 2013. Design of ultra-stable insulin analogues for the developing world. J. Health Spec 1:59—70.

[254] Balachandran, K., 2019. Cold chain and insulin: Should we follow the polio vaccine example? Indian Journal of Endocrinology and Metabolism 23(3):379, 379.

[255] Larger, E., 2005. Weight gain and insulin treatment. Diabetes and Metabolism 31(4):4S51—54S56.

[256] Chen, Z., Yu, R., Xiong, Y., Du, F., Zhu, S., 2017. A vicious circle between insulin resistance and inflammation in nonalcoholic fatty liver disease. Lipids in Health and Disease 16(1):1—9.

[257] Yang, Y., Petkova, A., Huang, K., Xu, B., Hua, Q.-x., Ye, I.-J., et al., 2010. An Achilles' heel in an amyloidogenic protein and its repair: insulin fibrillation and therapeutic design. Journal of Biological Chemistry 285(14):10806—10821.

[258] McKay, D., Renaux, B., Dixon, G., 1985. The amino acid sequence of human sperm protamine P1. Bioscience Reports 5(5):383—391.

[259] Rostène, W., De Meyts, P., 2021. Insulin: a hundred year-old discovery with a fascinating history. Endocrine Reviews. https://doi.org/10.1210/endrev/bnab020.

[260] Owens, D.R., Zinman, B., Bolli, G.B., 2001. Insulins today and beyond. The Lancet 358(9283):739—746.

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