Growth Factor Production:
A Case Study of Pegfilgrastim

A Solution for Neutropenia caused by Myelosuppressive Chemotherapy Treatments

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Abstract (Ali & Minh)

Amgen's Pegfilgrastim, sold under the brand name Neulasta, was approved in 2002 by the Federal Drug Administration (FDA) for the treatment of neutropenia due to chemotherapy. Neulasta boosts neutrophil production as an active defense against neutropenia and febrile neutropenia (FN), both common side effects of myelosuppressive chemotherapy. Neulasta was preceded to market by a recombinant form of G-CSF as well as Neupogen. Since their patents expired in 2015 and 2013 respectively, several biosimilars have been manufactured and sold. Here, we introduce pegfilgrastim for neutropenia, describe its mechanism of action, production at small and large scales, and model Neulasta's economic impact on Amgen, and influence on decreasing neutropenia and FN costs. We did scale-up calculations assessing feasibility for a method for fed-batch production in *E.coli.* We also explore newer methods of production under biosimilars and highlight a perfusion process with yeast (*Pichia Pastoris*) that operates hands-free for a long time and enables on-demand production of formulated dose for easy deployment.

Introduction (Ali)

Motivation & Current Solutions

Cancer

All human cells are generated, develop into mature cells, and then die within finite life spans (Stranford et al., 2023, p.768). Critical for regulating and maintaining a constant and proper number of every cell type in the body, this cell life cycle provides space for new cells to proliferate and also ensures that cellular information is passed on (Stranford et al., 2023, p.768). However, when a cell stops following the normal cycle and refuses to undergo apoptosis, a build up of non-apoptotic, actively proliferating cells can form a mass, which can morph into malignant or invasive tumors, commonly referred to as cancer (Stranford et al., 2023, p.768). It is important to remove tumor cells because if they are permitted to persist, they will cause numerous problems leading to the host's eventual death.

The immune system's role in cancer has long been studied; association between cancer and the immune response was first identified in 1500 BCE when the ancient Egyptian Ebers Papyrus noted a connection between infections and swelling (Stranford et al., 2023, p.794). Cancer again captured medical interest when in 1891, William B. Coley experimented with injecting bacteria into malignant tumor cells as a means to fight bone cancer (Stranford et al., 2023, p.784). This was regarded as one of the first attempts in relatively recent times to eliminate malignant tumors. Now, many modern cancer treatments exist, such as surgery, hormonal therapy, immunotherapy, radiotherapy, personalized therapy, bone marrow transplants, or chemotherapy (Stranford et al., 2023, p.783-784). And while some of these treatments can be very effective in eradicating cancer, some are less targeted and function as blunt instruments, damaging healthy cells as well.

Chemotherapy

While there are multiple cancer treatment modalities, chemotherapy has become perhaps the most common and well-known process. Depending on the type of chemotherapy, there are typically three main mechanisms of action. First, chemotherapy drugs can disrupt the cell division process rendering it impossible for the cancerous cells to proliferate. Second, chemotherapy can cause cell death by directly damaging the DNA or RNA of the specific cancer cells or by interfering with the production of essential proteins. Lastly, chemotherapy drugs can trigger cancer cell targeted apoptosis (McDonagh, 1916).

Chemotherapy is efficient at rapidly eliminating proliferating cells within the bone marrow and bloodstream, but horrible at targeting only malignant cells (McDonagh, 1916). Malignant (cancer) cells tend to be immune to apoptotic signals. They also tend

to ignore other immune signals, thus enabling proliferation at an alarming rate. Chemotherapy is efficient at killing rapidly dividing cells, which makes it an effective cancer combatant tool (McDonagh, 1916). However, as a by-product, these drug regimens also kill other non-malignant rapidly proliferating cells such as oral, GI tract, hair cells, and platelets (Kumar et al., 2018; Stranford et al., 2023). So, chemotherapy is efficient at rapidly eliminating proliferating cells within the bone marrow and bloodstream, but horrible at targeting only malignant cells (McDonagh, 1916). A dramatically impacted cell-type are white blood cells, a family of immune cells that play an integral role in preventing infection and illness from foreign pathogens or antigens (Stranford et al., 2023). The elimination of such healthy, rapidly dividing cells causes significant adverse impacts on the human body, by increasing susceptibility to infection by drastically hampering the body's immune system's ability to do its job (Stranford et al., 2023). In order to safely and rapidly restore a healthy white blood count in chemotherapy patients, drugs such as Neulasta can be critical life-savers, and are best if taken concurrently with chemotherapy.

Neutrophils

Granulocytes are an immune cell type that originate in the bone marrow and circulate within the bloodstream. They are a part of the innate immune response, often being the first cell type to respond (Stranford et al., 2023, p.38). Granulocytes are composed of four major cell types: neutrophils, eosinophils, basophils, and mast cells (Stranford et al., 2023, p. 38). In adults, neutrophils comprise the majority of granulocytes (70%) circulating your bloodstream (de Bont et al., 2019; Stranford et al., 2023, p. 38). Neutrophils are differentiated from hematopoietic stem cells (HSC) in the bone marrow. Once progenitor neutrophils are created, they are released into the peripheral blood stream to circulate around the body for 7-10 hours before moving into the body's various tissues (Stranford et al., 2023, p. 38).

When circulating in the body, neutrophils serve as the first line of defense against invading pathogens (de Bont et al., 2019). This defense consists of three primary mechanisms: degranulation, which is the release of cytotoxic cytokines from granules, phagocytosis, and NETosis (de Bont et al., 2019; Stranford et al., 2023). Neutrophil extracellular traps (NETs) are filaments that extend out 10-15 times the size of the neutrophil to trap bacteria, fungi, & parasites (Stranford et al., 2023, p. 153). NETosis is the process by which neutrophils create a sticky NET to catch pathogens by expelling their chromatin covered with antimicrobial proteins (de Bont et al., 2019). When chemotherapy eradicates malignant tumor cells and granulocytes, this causes the patient to become susceptible to infections and experience clinical side effects.

Neutropenia & Febrile Neutropenia

Neutrophil levels can be measured as part of the Complete Blood Count (CBC) differential, which delineates the total number of each type of blood cell present in the blood (*WebMD*, n.d.). Neutropenia, a common side effect of myelosuppressive chemotherapy, is a condition characterized by a low neutrophil cell count, which can be measured by the Absolute Neutrophil Count (ANC) (*WebMD*, n.d.). A neutrophil level is considered normal when it is in the range of 1,500 to 8,000 neutrophils per microliter (*WebMD*, n.d.). The Infectious Disease Society of America defines neutropenia as an ANC of 500-1000 cells per microliter with an anticipated decline in neutrophil count to less than 500 cells per microliter in less than 48 hours (Lucas et al., 2018). With a low neutrophil cell count, the immune system isn't able to fight foreign pathogens, which can lead to a patient's greater susceptibility to infections, especially bacterial. Patients who develop post-chemotherapy febrile neutropenia (FN) have been discovered to have a greater than 50% chance of developing an infection (Bodey et al., 1966).

Febrile neutropenia (FN) is characterized by the presence of neutropenia in conjunction with a fever (Lucas et al., 2018) and is another disease that also occurs post-myelosuppressive chemotherapy treatments. This fever, which is classified as an oral or tympanic temperature of 38.3℃ or greater for at least one consecutive hour (Lucas et al., 2018), can be seriously detrimental as 80% of those with FN are hospitalized (risking further exposure to infectious diseases). This hospitalization with a stay of 7 - 10 days places a significant burden on the U.S. healthcare system (Lucas et al., 2018; Tai et al., 2012). In 2012, Tai et al. evaluated the costs of both pediatric and adult neutropenia and FN patients. For pediatrics, the mean hospital stay lasted 8.5 days, averaging a mean cost of $26,000 per stay. For adults, the average length of stay was 9.6 days, accruing an average cost of $24,770 per stay (Lucas et al., 2018; Tai et al., 2012). Those hospitalized for FN also have a significantly higher mortality rate when compared to patients with neutropenia. This is most likely because even though only 30% of FN patients have identifiable infections, it is an infectious process that leads to mortality (Karimi et al., 2018). Those who are hospitalized for FN have mortality rates of 0.4-3% for pediatric cancer patients, 2.6%-7% for adults with solid tumors, and 7.4% for adults with hematologic malignancies (Boccia et al., 2022).

Granulocyte Colony-Stimulating Factor (G-CSF)

Granulocyte colony-stimulating factor (G-CSF) is a naturally occurring protein that stimulates the bone marrow to produce more neutrophils (Panopoulos et al., 2008). With natural G-CSF increasing neutrophil production and activity, the immune system can improve its defense against infection, and thus reduce the risk of FN (Averin et al., 2021; Panopoulos et al., 2008). This discovery spurred investigations in the 1980s into producing synthetic G-CSF. First, in 1986, Souza et al. investigated whether G-CSF had an effect on leukemic-myeloid cells. They found that human G-CSF could potentially inhibit myeloid leukemia cell growth, as demonstrated in a murine myeloid leukemic cell

model, by induction of terminal differentiation (Souza et al., 1986). Then, Cohen et al. (1987) implanted osmotic pumps containing *E. Coli* derived human G-CSF into hamsters. What they found was that within three days, there was a 10-fold increase in neutrophil-specific granulocyte counts and 4-fold increase in leukocyte counts. This increase continued with daily injections of the *E. Coli* derived human G-CSF (Cohen et al., 1987). In 1988, Grabrilove et al. conducted a Phase I clinical trial investigating the administration of a recombinant G-CSF in 22 humans undergoing transition cell carcinoma chemotherapy treatments. This drug was administered before the chemotherapy treatments. They saw a 1.8-12 fold increase in neutrophil count, depending on the dose of recombinant G-CSF provided (Grabrilove et al., 1988). Then, in 1991, a phase III clinical trial study was published in support of the use of a recombinant methionyl G-CSF drug, developed by Amgen, as a means to prevent fever, one of the five major signs of infection, in patients after receiving chemotherapy for small-cell lung cancer (Cohen et al., 1987; Gabrilove et al., 1988; Souza et al., 1986; Stranford et al., 2023). This recombinant methionyl G-CSF drug has been demonstrated to increase neutrophil production in patients with advanced neoplasms and reduce the magnitude of chemotherapy-induced neutropenia (Bronchud et al., 1987; Cohen et al., 1987; Gabrilove et al., 1988; Morstyn et al., 1988; Souza et al., 1986).

Crawford et al.'s (1991) double-blind phase III clinical trial investigated recombinant methionyl G-CSF in 211 patients (101 G-CSF and 110 placebo). Their results indicate that this recombinant methionyl G-CSF drug, when administered as an adjunct to chemotherapy for patients with small-cell lung cancer, significantly reduces their incidence of FN, as well as incidence rate, duration, and severity of neutropenia (Crawford et al., 1991). In 1995, Katano et al. investigated G-CSF's impact on chemotherapy-induced oral mucositis, finding that it was an effective preventative measure. In 2021, Averin et al. studied the response of patients suffering from metastatic cancer (e.g. breast cancer, colorectal cancer, lung cancer, and non-hodgkin's lymphoma) to G-CSF prophylaxis as well as the inherent risk of acquiring FN. Averin et al. (2021) found that even though there are many who are deemed eligible to receive prophylactic G-CSF, not everyone who is eligible does. There was a demonstrated higher prevalence of FN for participants who didn't receive prophylactic G-CSF during chemotherapy treatments compared to those who did (Averin et al., 2021).

All of these different studies support the use of G-CSF in the replenishment of neutrophils and illustrate that extensive investigation into G-CSF's involvement in neutrophil stimulation was conducted before the development of G-CSF biosimilars, including Neupogen and Neulasta.

Neulasta

Neulasta is a prescription medicine developed by Amgen and approved by the FDA in 2002. It is taken in conjunction with chemotherapy as a means to help reduce the chances of infection due to a low white blood cell count (*Neulasta Onpro*, 2024). It is

a later iteration or biosimilar to the recombinant G-CSF, in that both drugs were designed to stimulate the bone marrow into producing more neutrophils.

Neulasta is administered as an intravenous (i.v.) injection at a dose of 6mg/0.6mL contained within a prefilled syringe (*Neulasta Onpro*, 2024). This pre-filled syringe comes in a package with the on-body injector (*Neulasta Onpro*, 2024). This packaging and administration system makes it easy and possible for patients to administer the drug at home, reliably. Like all medications, Neulasta has some serious side effects that include but are not limited to, bone, arm, and leg pain, spleen ruptures, Acute Respiratory Distress Syndrome (ARDS), allergic reactions, Sickle-cell crisis, Glomerulonephritis, Leukocytosis, Thrombocytopenia, Capillary Leak Syndrome (CLS), Myelodysplastic syndrome, Acute Myeloid Leukemia (AML), and Aortitis (*Neulasta Onpro*, 2024). These side effects can be seriously detrimental or fatal, so it is important to fully understand the seriousness of taking Neulasta.

Product Description (Noorul)

Mechanism of Action

Pegfilgrastim is a 0.6mL prefilled syringe with 6mg recombinant human G-CSF protein with a 20kDa PEG molecule covalently attached to the N-terminal methionyl residue of the recombinant protein.

G-CSF, expressed by the gene *CSF3*, is a signaling protein in humans. It is recognized by granulocyte colony-stimulating factor receptor (GCSFR), encoded by the gene *CSF3R,* on cell surfaces. G-CSF activates a signaling cascade promoting neutrophil differentiation in granulocyte precursor stem cells and proliferation of neutrophils. G-CSF is a cytokine. Cytokine signaling is the first line of chemical defense against microbial invasion, recruiting neutrophils and natural killer cells to circulate in blood, interstitial spaces and epithelial surfaces. G-CSF increases neutrophil mobilization and maturation, so rh-GCSF, or filgrastim, has been used in clinical practice to prevent and treat neutropenia.

Structure

Filgrastim, or rh-GCSF, is a water-soluble 175 amino acid protein with a molecular weight of 18.8 kilodaltons (kDa). Filgrastim is obtained from the bioreactor cultivation of a strain of *E. coli* transformed with a genetically engineered plasmid containing the human G-CSF gene (*CSF3*). To produce pegfilgrastim, a 20 kD monomethoxy-polyethylene glycol (PEG) molecule is covalently bound to the N-terminal methionyl residue of purified filgrastim. Filgrastim and G-CSF are recognized by G-CSF receptors (GCSFR) on cell surfaces of neutrophils and granulocytes. GCSFR is a single transmembrane protein composed of multiple extracellular and intracellular functional components (Figure 1). The intracellular portion contains regions essential for

proliferative signaling and differentiation. The extracellular cytokine receptor-homologous (CRH) domain has a WSXWS motif to bind G-CSF and initiate signaling (Figure 2).

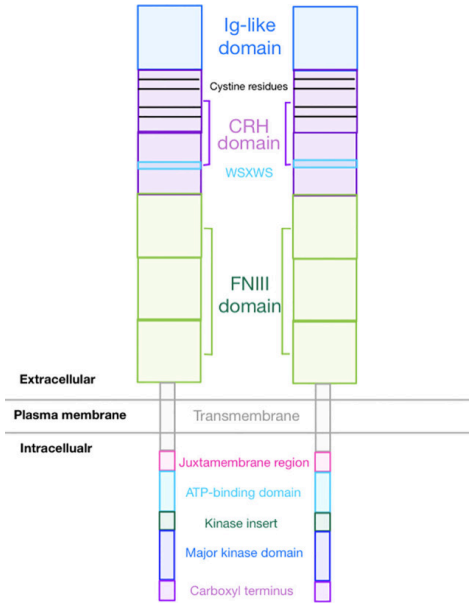

Figure 1: Structure of GCSFR receptor on cell surface. Immunoglobulin (Ig)-like domain, a cytokine receptor homologous (CRH) domain, and three fibronectin type III (FNIII) domains are in the extracellular region.

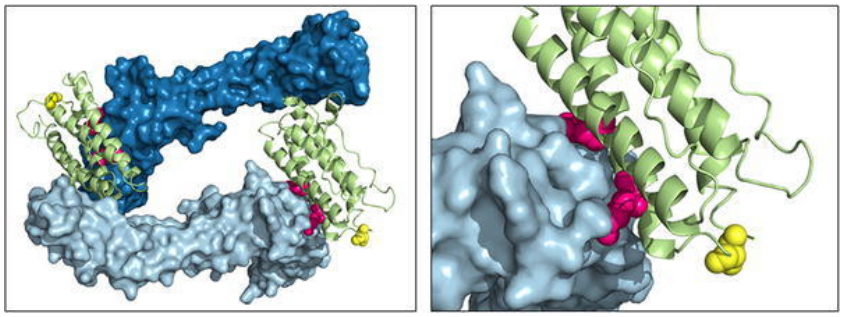

Figure 2: G-CSF (green) binding to GCSFR (blue and light blue). N-terminus atoms (yellow) where PEG molecule attaches are far from binding site lysines 16 and 23 (pink).

Upon binding of G-CSF to its receptor GCSFR, cellular proliferation is enhanced by a signaling cascade propagated by many factors, among which are Src and a tyrosine kinase protein, Janus Kinase (JAK). This results in the downstream transcription events of the signal transducers and activators of transcription (STAT) family: STAT1, STAT3 and STAT5 are implicated in promoting neutrophil proliferation [21] and granulocyte stem cell differentiation into neutrophils (Figure 3). G-CSF demonstrated proliferative signal transduction and immunomodulation properties in studies done using recombinant G-CSF produced from genetically engineered *E. coli.*

GCSFR signaling has also been found to be increased in multiple cancers as compared to expression levels in healthy cells further demonstrating its proliferative capacity.

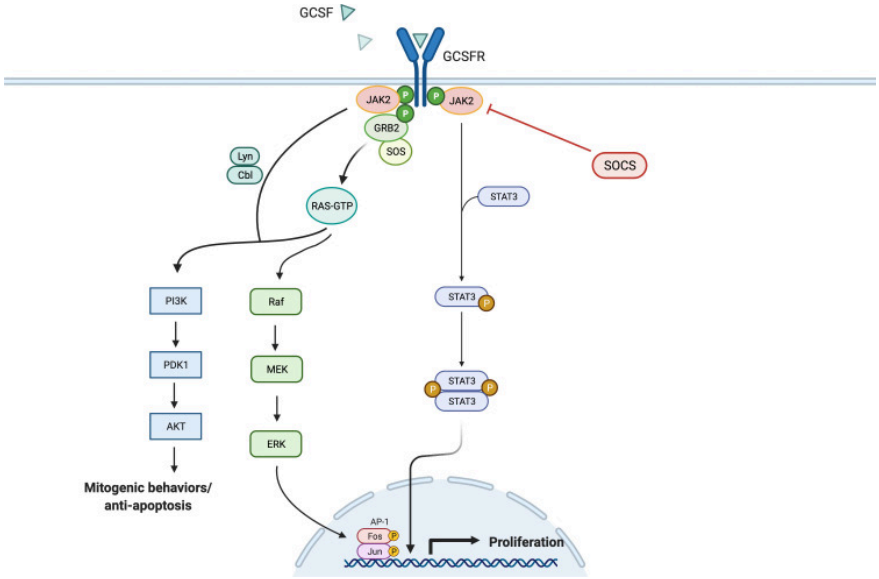

Figure 3: Overview of GCSFR signaling pathways. Pegfilgrastim, or pegylated recombinant G-CSF, binds to GCSFR and promotes neutrophil differentiation and proliferation functioning as a growth factor through JAK and STAT pathway activations.

Pegfilgrastim requires lower doses

G-CSF is cleared in humans by two mechanisms: renal clearance, and neutrophil clearance. Renal clearance filters G-CSF from blood in kidney glomeruli. Neutrophil clearance degrades the G-CSF and GCSFR complex intracellularly. Due to a decreased neutrophil content, renal clearance dominates clearance of G-CSF from blood in neutropenia. Filgrastim, a previous iteration of pegfilgrastim consisting solely of recombinant human G-CSF without the covalently attached PEG molecule, was developed by Amgen, approved by the FDA for neutropenia in 1991 and sold in the US under the brand name Neupogen. A major limitation of filgrastim is renal clearance. Glomerular filtration of G-CSF necessitates higher drug doses, increasing treatment cost. Filgrastim requires a daily dose compared to pegfilgrastim with one dose required per chemotherapy cycle. Pegfilgrastim with its covalently attached PEG molecule is larger in size, allowing it to bypass renal clearance. Pegfilgrastim is only degraded by neutrophil clearance which occurs when neutrophils increase in number, thus the drug clears on its own when no longer needed. Since G-CSF is also cleared by neutrophils, bypass of renal clearance by pegfilgrastim does not pose a safety issue. Bypass of renal clearance translates into lower doses needed for the same clinical effectiveness.

Host Cell Lines (Ali)

Neulasta is synthesized using recombinant DNA technology in *E. coli*. Chinese Hamster Ovarian (CHO), mouse myeloma NS0, human kidney HEK293, and *Pichia Pastoris* cells have also been used for recombinant production.

Amgen uses *E. coli* bacteria transfected with the human G-CSF gene for filgrastim (Neupogen) and pegfilgrastim (Neulasta) because both contain G-CSF. The amino acid sequence is identical to the natural G-CSF that occurs in our bodies. Neulasta is non-glycosylated due to production in bacteria. The absence of glycosylation has been shown to have no clinical difference.

Drug Process Description

Discovery & Cell Line Development

GCSF plays a crucial role in regulating the development of antibacterial white blood cells called neutrophils. Initially, murine G-CSF was isolated from fluid surrounding tissues of animals injected with bacterial cell wall extract, with its effects on hematopoietic cells (Nicola et al.). Later, Human G-CSF was isolated from a medium conditioned by the 5637 human bladder carcinoma cell line in 1985 (Welte et al.), and subsequently from the squamous carcinoma cell line CHU-2 (Nomura et al.). Through the use of recombinant protein technology, Amgen researchers engineered an expression vector to produce ample quantities of huG-CSF for both in vitro and in vivo biological investigations.

The construction of this vector involved a three-step DNA ligation process, incorporating a modified ts-runaway plasmid with an XPL promoter, the hG-CSF gene, and a synthetic DNA fragment containing an initiation codon followed by the sequence encoding the mature form of huG-CSF. E. *coli* containing the resulting expression plasmid were cultured and induced to synthesize recombinant huG-CSF (rhuG-CSF).

While hG-CSF purified from tissue culture displayed an apparent molecular weight (MW) of 19,600, the E. *coli*-produced rhG-CSF exhibited an apparent MW of 18,800. The deduced amino acid sequence predicts a MW of 18,700, closely matching the measured MW of rhG-CSF, indicating a post-translational modification, O-glycosylation, of native hG-CSF (Souza et. al). Despite the superior specific activity of glycosylated G-CSF, the non-glycosylated G-CSF drugs have accounted for almost the entire global market thus far due to the economic advantages of using *E. coli* expression system to produce recombinant protein such as cost-effectiveness, rapid growth rate, and high yield of protein production.

The non-glycosylated form of rHuG-CSF led to the approval of Filgrastim (r-metHuG-CSF) for patient use in the US in 1991. Another form derived from Chinese hamster ovary cells (CHO), lenograstim, was approved for use in Europe in 1993. Initially used to treat neutropenia caused by cancer chemotherapy and prevent associated infections, G-CSF has since found applications in bone marrow

transplantation, congenital neutropenia, AIDS, leukemia, anemia, and mobilizing stem cells for transplantation. Various forms of rHuG-CSF are available globally, including filgrastim (Neupogen®), lenograstim (Granocyte®), and KW-2228 (Nartograstim®).

Current Process of Pegfilgrastim Production

The process workflow of therapeutic recombinant human G-CSF (rHuGCSF) production includes upstream and downstream processing. Upstream processing involves thawing cells from the working cell bank followed by cell culture expansion via bioreactors. Downstream processing involves the isolation and purification of GCSF, PEG conjugation, formulation, and final finish.

The production of recombinant therapeutic proteins using microorganisms as host systems is often difficult because the overexpression of recombinant proteins leads to the formation of inclusion bodies (IBs) that are insoluble aggregates. IBs formation can arise from the accumulation of partially unfolded forms of recombinant proteins and produce biologically inactive forms within cells, requiring costly, additional downstream steps to make the protein biologically active for further purification.

While platform processes for monoclonal antibodies have existed for more than a decade, development of an integrated continuous manufacturing process for recombinant proteins have received relatively scant attention. The continuous process takes inclusion bodies as the feed material and produces the final unformulated drug substance. Researchers recently developed a novel coiled flow inverter based reactor (CFIR) for continuous refolding and the incorporation of surge vessels for continuous integration with the following chromatography steps (Kateja et al., 2017; Sharma et al., 2016). Continuous PEGylation of therapeutic proteins has been explored using advanced technologies such as hollow fiber membrane reactors and on-column counter-current chromatography. These methods offer process intensification and increased productivity, with the chromatographic approach demonstrating a threefold productivity boost. However, despite their promise in enhancing selectivity and productivity, the complexity of these techniques has hindered their widespread adoption in industry. Additionally, limited data exists on factors like fouling, back pressure, and lifetime, partly due to the inherent properties of the PEG reagent used in these processes (Kateja et al., 2020).

Upstream Processing

As is the case with most biotech companies, Amgen did not disclose any cell expansion and production processes for rHuGCSF. Reviewing past literature, the primary goal of fermentation is the cost-effective production of bioproducts, so to enhance the volumetric productivity, high cell concentration and productivity are desired. This is often achieved through fed-batch cultivation methods (Matsui et al., 2006; Chung et al., 2006). Amgen utilizes a two-tiered cell bank consisting of a master cell bank

(MCB) which can be used to create a working cell bank (WCB). In short, the upstream process comprises: the thawing of two vials from WCB which are then expanded via shake flasks and bioreactors until seeding in the production scale fermenter. Exponential feeding profiles derived from Monod kinetics are the most common feeding strategy applied in industry. The exponential-feeding method has been developed to allow cells to grow at constant specific growth rates. It also provides the advantage that acetate production can be minimized by controlling the specific growth rate below the critical value of acetate formation (Babaeipour et al., 2017). To induce the expression of GCSF in E. *coli, Isopropyl* β-D-1-thiogalactopyranoside (IPTG) is used. Specific ways to scale up fed-batch cultivation methods will be described in the scale-up section below.

Downstream Processing

Just like upstream processing, Amgen did not disclose any details of downstream processing. The goal of downstream processing is to create a highly pure product. Reviewing past literature, a scalable industrial process for isolation and purification of granulocyte colony stimulating factor (G-CSF) is proposed: To purify rHu-GCSF produced by high cell density fermentation, separate the soluble and particulate fractions using centrifugation. Then further lyse the E. *coli* cells in a lysis buffer at pH 7-9. Isolate inclusion bodies using a laboratory scale sonicator and high pressure cell homogenizer. Next, solubilize the G-CSF by incubating the isolated inclusion bodies, and then, refold the solubilized G-CSF by diluting it into a refolding buffer for 12 to 28 hours at room temperature, which contains oxidized glutathione (GSSH) and cystamine. Then add sodium acetate, hydrochloric acid, and orthophosphoric acid to arrive at a pH in the range of 3.0 to 5.5. Then clarify the solution using filtration and purify the solution by first cation exchange chromatography (CEX) and then hydrophobic interaction chromatography (HIC) (Babaeipour et al., 2015).

Next, conjugate the purified G-CSF with PEG to produce mono-pegylated G-CSF by incubating a stirred conjugation reaction mixture comprising a conjugation buffer solution, a PEG, and 20 mM of a reducing agent (sodium cyanoborohydride) for 12 to 18 hours at room temperature. The conjugation buffer solution contains sodium acetate. Then purify the mono-pegylated G-CSF by cation exchange chromatography, which contains a stationary and a mobile phase. The stationary phase uses MacroCap-SP where the mode of elution is linear gradient. Next use ultrafiltration to obtain a composition comprising 9-12 mg/ml mono-pegylated G-CSF. During fill and finish, dilute the solution to the target concentration of 6 mg/0.6mL. A proposed representation of the upstream and downstream processing is depicted below in Figure 4 (Raghuwanshi, A et al., 2015).

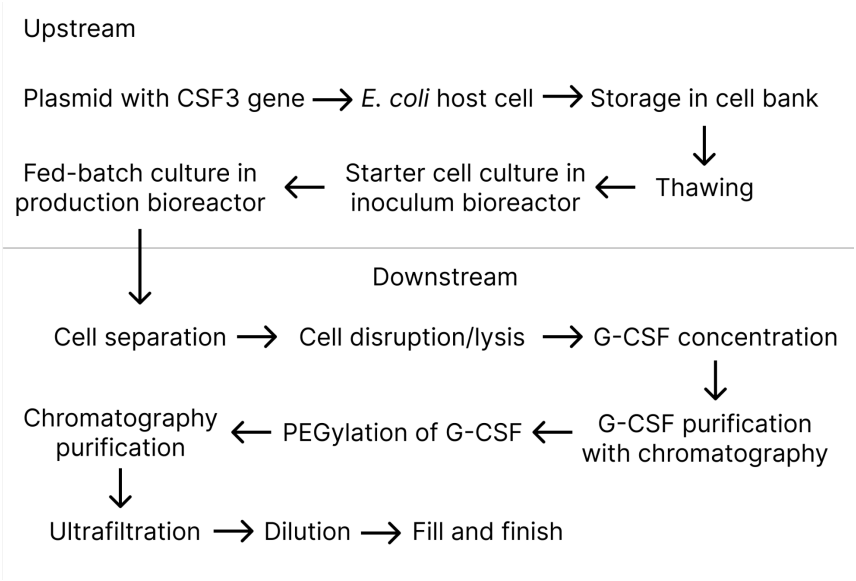

Figure 4: Process flow diagram for pegfilgrastim production. This process remains the same for filgrastim production.

Scale Up (Minh)

Scaling up efforts to achieve an appropriate economic scale for production of peg-G-CSF starts with the production of G-CSF itself. Scaling up the fed-batch process can be done by a feeding strategy based on specific growth rate. Feeding rate was derived after carbon source depletion based on this equation: $F(t).S(t) = [\mu/Y_{x/s}+m]\ X_{to}.V_{to}\ \exp[\ \mu.(t-t_0)]$ (Choi JH, 2006). The rate of feeding takes into account not only concentration of glucose (monitored manually), constant volume of culture (by using concentrated feed), but also is an exponential of specific growth rate. This ensures that glucose concentration remains near zero without fluctuations (due to constant specific growth rate), and the feeding scheme ensures that the metabolism of the culture stays constant (Babaeipour et al., 2017).

However, with exponential feeding strategy, we run into problems maintaining DO because the cells are in a high specific growth rate. By maintaining the geometry of the vessels and the DO at higher than 20% based on changing impeller speed, and using air with enriched oxygen, the author was able to achieve a titer of 22g/L and 19g/L pre purification when scaling up to a 1L and 8L scale respectively. We did our own calculations to determine the operational parameters for the scale-up from 1L to 10L as well as a larger scale (1L to 100L).

Babaeipour et al (2017) stated that standard size bioreactors that have a working volume of 1L and 8L were used. We have found specifications for Eppendorf bioreactors, with working volumes of 1L, 10.5L, and 100L respectively and determined the geometry as well as corresponding impeller speed that we need to operate at (Table 5). Given that we only have the power number (Np) for constant gassing at 1.5vvm, we

assumed that the author maintained DO constant by increasing oxygen content in the input air and not increasing total air input. Since the author kept the bioreactor geometrically constant and the power per unit volume (P/V) constant, we can determine a P/V that allows the highest tip speed, which can be used to determine our highest possible mixing speed to allow best gas circulation (Tip speed = V/(NDi)). This can be calculated based on the previous assumptions. Figure 5 utilizes the same scale-up strategies on commercially available Eppendorf bioreactors. We chose our P/V to be 2.42 kW/m^3, which is the highest P/V with the highest corresponding tip speed achievable, empirically derived by Eppendorf **(Figure 6)**. We determined impeller speed ($Ni_{calculated}$) to be 820 rpm, 501 rpm, 503 rpm respectively for the 1L, 10L, and 100L scales (Eq 1,2,3,4,Appendix B)

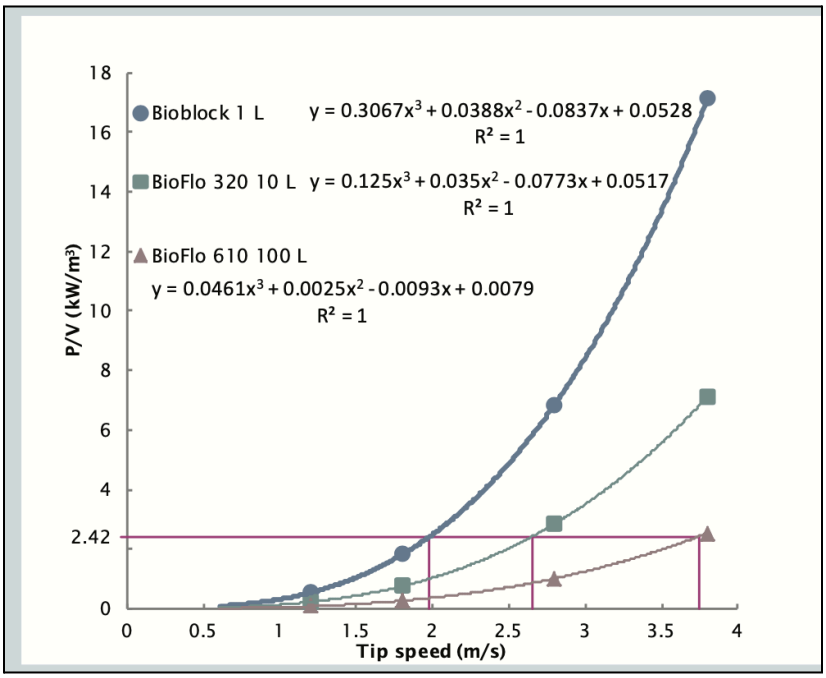

Figure 6: Determining the constant P/V values for scale-up under 1.5 vvm of airflow

	Eppendorf Bioblock 1L	**Eppendorf BioFlo 10L**	**Eppendorf BioFlo 610 100L**
Working Volume V (m^3)	1 L = 0.1 m^3	10.5 L = 0.0105 m^3	150L = 0.15m^3
P/V (kWm^{-3})	2.42 = 2420 Wm^{-3}	2.42 = 2420 Wm^{-3}	2.42 = 2420 Wm^{-3}
N_{max}	1600 rpm = 26.7 s^{-1}	1200 rpm = 20 s^{-1}	500 rpm = 8.3 s^{-1}
$N_{calculated}$	13.6 s^{-1}= 820 rpm	10.0 s^{-1} = 601 rpm	8.40 s^{-1} = 503 rpm
Np at 1.5vvm	4.6	5.9	5.0

Liquid height H_{max}	136 mm = 0.136 m	323 mm = 0.323 m	904 = 0.904 m
Di/Dt	0.4	0.4	0.4
H_{max}/Dt/Di	0.7	0.8	0.8

Table 1: Scaling up of example bioreactors from bench scale to pilot scale
*Values for Vmax, Di, Dt, Nmax taken from manufacturers specification for bioreactors
*Np at 1.5 vvm empirically derived by Eppendorf (Kononova et al,, 2010)

The scale-up method is based on constant geometric bioreactor sizing and DO concentrations coupled with exponential feeding strategy, which provides the ability to scale up without forgoing productivity. This is done by maintaining efficient gassing and high specific growth rate, but producing low levels of waste (due to glucose being constantly near zero).

Comparison of Alternative Processes (Minh)

A biosimilar product is a biologic that is highly similar to a pre-existing FDA - approved biologic product (reference product). A biosimilar must have no clinically meaningful differences in safety or effectiveness from the reference product. The only differences allowed in a biosimilar product are minor differences in clinically inactive components (Pfizer, 2021). As the patent for Neupogen (G-CSF) expired in December 2013 and the patent for Neulasta (peg-G-CSF) expired in October 2015 in the U.S., there have been many biosimilars since approved by the FDA (Table 2) (Steward, 2015).

Neupogen (filgrastim) Biosimilars

Name	Regulatory Designation	Company Name	FDA Approved
Releuko (filgrastim-ayow)	Biosimilar	Kashiv BioSciences, LLC	February 25, 2022
Nivestym (filgrastim-aafi)	Biosimilar	Pfizer Inc.	July 20, 2018
Zarxio (filgrastim-sndz)	Biosimilar	Sandoz Inc.	March 6, 2015

Neulasta (pegfilgrastim) Biosimilars

Name	Regulatory Designation	Company Name	FDA Approved
Stimufend (pegfilgrastim-fpgk)	Biosimilar	Fresenius Kabi USA, LLC	September 1, 2022
Fylnetra (pegfilgrastim-pbbk)	Biosimilar	Amneal Pharmaceuticals, Inc.	May 26, 2022
Nyvepria (pegfilgrastim-apgf)	Biosimilar	Pfizer Inc.	June 10, 2020
Ziextenzo (pegfilgrastim-bmez)	Biosimilar	Sandoz Inc.	November 4, 2019
Udenyca (pegfilgrastim-cbqv)	Biosimilar	Coherus BioSciences, Inc.	November 2, 2018
Fulphila (pegfilgrastim-jmdb)	Biosimilar	Mylan N.V.	June 4, 2018

Table 2 : Approved biosimilars for G-CSF and peg-G-CSF in the US

Biologics differ from small molecule drugs due to difficulties in identifying the exact structure of manufactured biologics. Since they are made in live cells, we cannot do a simple Proton nuclear magnetic resonance (H-NMR) to exactly identify the molecules' structure. Hence, FDA- approved biologics must retain their bioprocess for the length of the patent.Therefore, with the production of biosimilars, a lot of innovations in bioprocessing happen concurrently. Many improvements in the production process of

G-CSF, which is needed to make peg- G-CSF has been made: A change of host from *E.coli* to *Pichia Pastoris* enables the molecule to be made extracellularly instead of intracellularly. A change of production method from fed-batch to perfusion enables continuous processes to reduce downtime in between batches. This section will go in depth about how optimization of the host, molecule, and a novel continuous perfusion process lead to the ability to produce G-CSF on demand at high productivity (Crowell, L., Lu, A., Love, K. et al, 2018).

The potential for *Pichia Pastoris* as an alternative host for G-CSF stems from a disadvantage of producing G-CSF in *E.coli*. The production of G-CSF in *E.coli* is intracellular, meaning that the proteins are made and accumulated in inclusion bodies. Due to G-CSF being aggregation prone, the accumulation of it in inclusion bodies made purification yield to be 40% of total produced protein (Kononova, N.V., Bobruskin, A.I., Kostromina, T.I. et al, 2010). With *Pichia Pastoris*, the protein can be secreted into the cultured medium, which decreases aggregation. The yeast also secretes a consistent profile of host-cell proteins along-side the recombinant protein of interest during fermentation (Matthews, C.B. et al 2017). This feature of the host allows secretion of high level of initially pure product (80%), which can be leveraged into creation of a purification process that is integrated into the system, leading to a continuous process that has both upstream and downstream processing ability (Timmick, S.M. et al, 2018).

The InSCyT system was developed to consist of a perfusion bioreactor (USP) connected to a purification system (DSP), and connected to formulation (TFF) (Figure 7a,b,c). Crowell et.al used custom-modified Multifors 2 NW70 benchtop bioreactors equipped with 0.75 L flat-bottomed glass vessels. The impellers are magnetically coupled, which consists of two Rushton impellers. The operating system is connected to pH, dissolved oxygen (DO), and temperature probe, which is inside of the bioreactor, allowing operators to check on the system online. InSCyT uses disposable custom tubing assembly, which is gamma radiated, ensuring no process contamination. The deployment of the disposable tubing assembly helps reduce the chance of contamination, which can happen during cleaning of stainless steel bioreactors. InSCyT can also be run hand-free with automatic flow-through in between modules.

Noticeably, upstream processes require a similar amount of effort for fed-batch and perfusion when producing G-CSF, but the difference lies in down-time. Sterilizing and re-inoculating fed-batch leads to lower productivity in the long run when compared to perfusion, which can be run continuously. Furthermore,the purification process for *E.coli* requires a lot of hands-on effort (Figure 8). Compared to flowing straight through a cation exchange column, anion exchange column and hydrophobic charge-induction chromatography to formulation, traditionally, purification is hands-on with a lysis step, which utilizes a homogenizer, then we need to cycle through resuspension and ultracentrifugation steps. With this style of purification, man power is needed to move

the retentate through steps, then finally through an anion exchange column and cation exchange column.

The production process begins in strain construction, where G-CSF was codon-optimized to *Pichia Pastoris*, coupled with a truncated alpha-factor as signal sequence to enable secretion of recombinant protein. The bioreactor was charged with Rich Defined Medium (RDM) containing 4% Glycerol for the biomass accumulation phase, then inoculated with inoculum (Matthews et al., 2018). During the biomass accumulation phase, DO was kept at 100% and pH at 6.5, temperature at 25℃. At this stage, the perfusate is directed to waste. After 32 hours, induction occurred by switching media to RDM containing 3% Methanol. DO maintained at 25% with the same temperature and pH. After the media switch, the filtered perfusate from the culture was harvested and directed to pHam (pH adjustment module) to be pH adjusted to 5.5. This starts the purification process (Figure 8c).

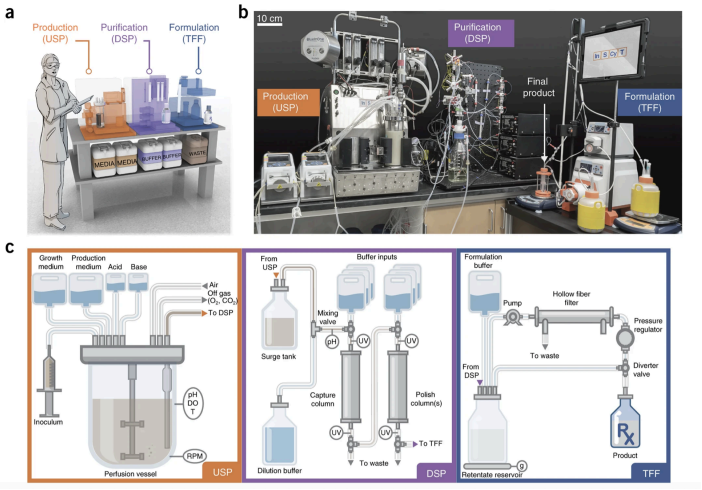

Figure 7: a. To-scale rendering of the InSCyT system. (b) Photograph of an operational InSCyT system. (c) Detailed schematic of the InSCyT system, including interactions between modules and key control points for the production (upstream processing, USP), purification (downstream processing, DSP) and formulation (tangential flow filtration, TFF) modules.

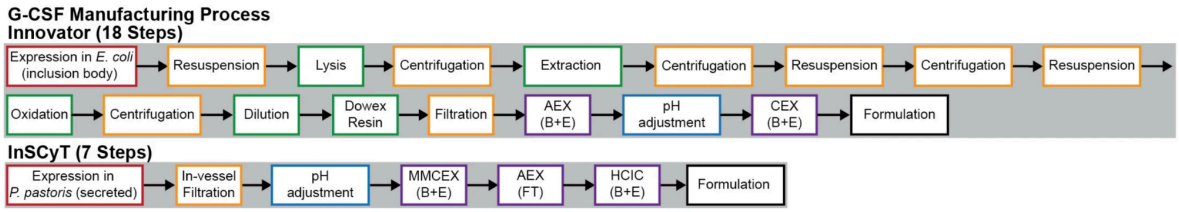

Figure 8: Process diagram of G-CSF Manufacturing process with fed-batch and process diagram of G-CSF manufacturing process with perfusion

The InSCyT system has been fully developed into a product by Sunflower Therapeutics. However, we would like to dive into the prototype that they ordered from Mutifors. We have obtained the data sheet from the manufacturer to calculate Reynold's number. Calculated Reynold's number is higher than 1000, the custom bioreactor is in turbulent flow, which ensures well-mixing (Eq 5, Appendix C).

In the InSCyT system, there are 2 Rushton turbines in the impeller. We also investigated the gas dispersion ability of the Rushton turbine.. By calculating the Froude Number in Equation 6, we can calculate that with the size of the provided turbine, one turbine can completely dispersed about 0.002 m^3 per minute (Eq 6,7,8, Appendix D). From the provided manufacturer specification, the gassing rate can be calculated to be about 0.001 m^3 per minute (Eq 9, Appendix D). Hence, a singular turbine can completely disperse the gas that is put into the bioreactor through the sparger. Due to the aggregation prone nature of the G-CSF molecule, we also looked into how much we can reduce shear without affecting gas dispersion. Using the gassing rate provided by the manufacturer, we calculated the lowest possible impeller speed to fully disperse the air provided. The lowest possible impeller speed is 340 rpm (Eq 10, Appendix D). Since the reported runs for the InSCyt system was not optimized, one possible future direction can be to reduce the impeller speed to 650 RPM - 700 RPM.

Total yield of the perfusion process is 30 mg/L/day, which scales with total time of the machine running. For a process that lasts 4 days, the production titer is comparable to titer previously reported in Kononova et al. (2010), which was 110 mg/L. This titer is still smaller than scaled-up fed-batch, but InSCyT can run continuously for 2 weeks.. Comparing two processes of making G-CSF prior to pegylation, the small InSCyT system is necessary for clinical trials and on-demand production of formulated protein. With a small-scale perfusion system, we can produce small batch trial drug doses with the same cost-saving as a scaled-up process. For production of drugs for sale, the company that commercialized InSCyT has a scaled up version (12000L) to solve the problem, but no paper is published about the machine.

Economics (Joe & Noorul)

This section focuses on the effect of Neulasta on Amgen's bottom line. Neulasta was an incremental addition to Amgen's existing production line of Neupogen. In terms of technology, an additional step was performed on purified G-CSF, already being produced at scale for use in Amgen's earlier product, filgrastim sold under brand name Neupogen. The additional step slightly increased costs due to an additional downstream steps of pegylation and subsequent purification, slightly raising Neulasta's cost over Neupogen. Even with slightly higher costs, Neulasta significantly affected Amgen's bottom line, being highly successful for neutropenia and febrile neutropenia. Growth in Neulasta sales more than made up for slowdown in Neupogen sales. Though both

drugs shared markets, Neulasta has significantly lower dosage compared to Neupogen which translated to buyers increasing their inventory 2002 onwards.

Exact data on Neulasta sales are missing as Amgen reports combined Neupogen and Neulasta sales 2003 onwards. Here, we report Amgen's potential market for neutropenia and Neulasta's effect on Amgen's balance sheet. We estimate Neulasta's cost of production and link it to sales.

2003 was the first full year in which Neulasta was sold. 1.33 million people were diagnosed with cancer in the US in 2003. Neutropenia is a significant challenge for anyone diagnosed with cancer, especially for those under chemotherapy. We assume 40% of all cancer patients require medicine against neutropenia, because chemotherapy is used against the majority of cancers. Pegfilgrastim is given once per chemotherapy cycle to combat neutropenia. There are 4-8 cycles in a chemotherapy treatment lasting a duration of 3-6 months. Pegfilgrastim dosage is one 6mg/0.6mL prefilled syringe. Based on these data, we estimate 532,000 cancer patients required 25.536 kg of pegfilgrastim in 2003. Pegfilgrastim was priced at $6,417/dose in 2022 and about $3,000/dose in 2003. In 2003, pegfilgrastim generated $1.2 billion in annual sales, reported by Amgen. 400,000 doses cost $1.2 billion at $3,000 a dose, totaling 3.5 kg of product assuming 70% efficiency in downstream processing. Amgen captured 10-14% of total market share for neutropenia growth factor biologics in 2003.

If a 100L fed-batch bioreactor costs $61 million to run a year in operating costs and produces 2g/L of product over 21 annual cycles, Amgen would need about 9 upstream production units every year to meet total pegfilgrastim demand. To meet its own demand of 3.5 kg per year, Amgen only needs 1 production unit. At $61 million operating costs generating $463 million in revenue in its first year (2002), Neulasta (pegfilgrastim) was well poised to take over Neupogen's declining market share. Neulasta improved upon Neupogen and was less expensive to develop since its development was not from scratch, and existing processes and infrastructure from Neupogen (filgrastim) could be repurposed. Revenue in 2003 from Neulasta and Neupogen combined increased 53% from Neupogen-only sales in 2002 even though Q4 2002 was the first quarter with Neulasta available. While sales of Neupogen have slowed, pegfilgrastim proved to be a blockbuster drug grossing $1.1 billion even in 2022.

Efficient production under the yeast perfusion process will decrease operating costs. A 47 L continuous (perfusion) process that produces 0.03 g/L of pegfilgrastim daily, is assumed to cost $32 million annually in operating costs. Production of 25,536 g of pegfilgrastim produced over 270 operating days in 67 perfusion systems will cost $2.14 billion. This figure is expensive compared to the $366 million operating costs calculated for the fed-batch process above but offers significant flexibility of scaling production to demand.

Future Improvements

The approvals of biosimilars increased market competition and led to a precipitous drop in pegfilgrastim prices of $134 every 3 months as seen in table # (Edward C. Li et al., 2021). Overall this represents a price decline

In recent years a new delivery method for pegfilgrastim has been released in the form of an on-body-injector which is attached to the patient during inpatient chemotherapy treatment and releases the drug at the appropriate time, roughly 27 hours post treatment, which enables consistent release timing The original delivery method is prefilled syringes given to a patient to self administer between 24 and 72 hours post treatment. With ease of self-administration, patients no longer need to return to the clinic. . A statistical analysis of available treatment data has shown that on-body-injectors did not substantially decrease the likelihood of FN or the total health care cost (A McBride et al., 2021). This is in contrast with other studies which have shown that 97.6% of patients using an on-body-injector received pegfilgrastim within the recommend 24-72 hours post treatment window for all treatments compared to only 63.1% of patients using prefilled syringes (M Metz et al., 2021).

Month, Year	Average Pegfilgrastim ASP	Breast Cancer $/QALY	NSCLC $/QALY	NHL $/QALY
July 2018	$4,554	$200,320	$113,942	$103,495
July 2019	$4,034	$178,152	$100,241	$89,918
July 2020	$3,483	$149,071	$82,267	$72,107
April 2021	$3,024	$124,845	$67,294	$57,269

Table 3: Values of pegfilgrastim average sales price and the dollar per quality-adjusted life years for the three most common cancer types which utilize pegfilgrastim treatment

Conclusions (Ali & Minh)

Neutropenia poses a large problem regarding hospitalizations as well as a financial burden on the U.S. market. It is a problem specifically for myelosuppressive chemotherapy patients as this chemotherapy treatment directly eliminates the neutrophils thus contributing to the onset of this disease.

Neulasta was created by Amgen in 2002 as a drug to be administered in conjunction with myelosuppressive chemotherapy treatments. Neulasta helps replenish neutrophils by stimulating the bone marrow to create more. Neulasta (Pegfilgrastim) is a pegylated form of Filgrastim (Neupogen). Amgen didn't disclose their cell expansion and production protocols, however, Neulasta is cultured in *E. coli*. In the biotech industry, *E.*

coli is a commonly used host cell line for its fast proliferation rate and simple nutrient requirements.

Neulasta reduced the amount of hospitalizations for Neutropenia, alleviating the financial burden on the U.S. economy. It was able to do this because Neulasta brought in revenue to the biotech industry with sales having reached $2.3 billion in the first three years it was on the market. Also, Neulasta was able to lower the medical costs of Neutropenia by reducing its occurrence. Combining Neutropenia's hospitalization costs with its downregulation in occurrences, the overall cost for the economy decreases. This is possible because Neulasta and its biosimilar's implementation brings revenue in and keeps more people out of hospitals.

The biosimilars further drove prices down when they were introduced to the market, making the treatments more accessible. Patent expirations allows innovations in process development with regards to host cell lines, manufacturing equipment, and scale-up method. This is because biosimilars are the same molecule, but with different process development. These biosimilars have been cultured in yeast cell lines as well as the original *E. coli*. Advances in production processes, both in research and development, upstream and downstream processes enhance productivity. This paves the way for on-demand production of critical biologics. With the applications of small scale perfusion, we can produce smaller quantities of drug with the similar low cost as the scaled-up processes. Overall, Neulasta provides the industry with the beginning of improvements to the biomanufacturing processes in scale-up capabilities as well as cost effectiveness.

Appendix

A. Calculation of drug quantity requirement for 2002:
   1.33 million new cancer patients x 40% with neutropenia x 8 doses per cycle on average x 1 cycle per year x 6mg per dose = 25,536 g

B. Calculation for impeller speed scaled up according to bioreactor size, keeping P/V constant

$$P/V = \frac{Np \times \rho \times N^3 \times Di^5}{V} \quad \text{(Eq. 1)}$$

$$For\ 1L:\ N = \sqrt[3]{\frac{(2420\ Wm^{-3})(0.001m^3)}{4.6 \times (1000kgm^{-3})(0.046m)^5}} = 13.6\ s^{-1} = 820\ rpm \quad \text{(Eq. 2)}$$

$$For\ 10L:\ N = \sqrt[3]{\frac{(2420\ Wm^{-3})(0.0105m^3)}{5.9 \times (1000kgm^{-3})(0.0844m)^5}} = 10.0\ s^{-1} = 601\ rpm \quad \text{(Eq. 3)}$$

$$For\ 100L:\ N = \sqrt[3]{\frac{(2420\ Wm^{-3})(0.15m^3)}{5 \times (1000kgm^{-3})(0.1651m)^5}} = 8.40\ s^{-1} = 503\ rpm \quad \text{(Eq. 4)}$$

C. Calculation of Reynold's number for InSCyT

$$Density\ of\ water\ at\ 25℃ = 997\ kg/m^3$$
$$Viscosity\ of\ water\ at\ 25℃ = 0.0089\ kg/ms$$
$$Impeller\ speed = 900\ RPM = 15\ s^{-1}\ *$$
*(Approximated from graph in paper that showed stirring speed)

$$Re = \frac{\rho \omega D^2}{\mu} = \frac{(997\ kg/m^3)(15s^{-1})(0.07\ m)^2}{0.0089\ kg/ms} = 8.2 \times 10^4 \quad \text{(Eq. 5)}$$

D. Calculation of gas flow for InSCyT

$$Froude\ Number\ (Fr) = \frac{Ni^2 Di}{g} = \frac{(15\ s^{-1})^2 (0.03\ m)}{9.8\ ms^{-2}} = 0.6887 \quad \text{(Eq. 6)}$$

For complete dispersion:

$$Gas\ flow\ number\ (Fl_g) = 0.2 \times \left(\frac{Di}{Dt}\right)^{0.5} Fr^{0.5}\ 0.2\left(\frac{0.03\ m}{0.07\ m}\right)^{0.5} 0.6887^{0.5} = 0.10866 \quad \text{(Eq. 7)}$$

$$Fl_g = \frac{Fg}{NiDi^3}$$

$$Fg = FlgNiDi^3 = 0.10866(15s^{-1})(0.03m)^3 = 4.4 \times 10^{-5} m^3 s^{-1} = 0.00264\ m^3 min^{-1} \quad \text{(Eq. 8)}$$

$$Specific\ gas\ rate\ (according\ to\ manufacturer) = 2\ vvmin^{-1}$$
$$Gassing\ rate = 2 \times 470mL\ min^{-1} \approx 0.001\ m^3 min^{-1} \quad \text{(Eq. 9)}$$

$$Lowest\ possible\ impeller\ speed = \frac{0.001m^3 min^{-1} \div 60s}{0.1088(0.03m)^3} = 5.68s^{-1} = 340\ rpm \quad \text{(Eq. 10)}$$

E. Calculations for pegfilgrastim earnings in 2003:
   $1.2 billion in annual pegfilgrastim sales (Amgen annual report, 2004) / $3,000/dose (assumed) = 400,000 doses
   400,000 doses * 6mg/dose = 2.4 kg of product
   70% downstream processing efficiency x total upstream filgrastim = 2.4 kg of product
   Total upstream filgrastim needed (recombinant G-CSF) = 3.5 kg
   Total pegfilgrastim and filgrastim needed in the market for neutropenia = 25.536 kg
   Market share of pegfilgrastim = 2.4 / 25.536 x 100 = 9.4%

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APA Format

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