UNIVERSITY OF OSLO

FACULTY OF DENTISTRY

Oscillations in mRNA-levels

Explored Through ErbB-ligands in Cisplatin-resistant Oral Squamous Cell Carcinoma

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Part 1: **Introduction**

1.1 Oral cancer is a leading cause of death

Cancer is a leading cause of death. In 2008, it caused 7,6 million deaths worldwide – a number which is estimated to reach 13,1 million in 2030. [1] Amidst the cancer-related deaths, oral cancer is ranked the eighth most common; its 5-year survival rate is only 50 percent. Over 90 percent of the oral cancers are, in fact, oral squamous cell carcinomas (OSCC). [2, 3]

This master thesis will focus on the work I completed together with my mentors Professor Trond S. Halstensen and Ph.D-candidate Gao Jian. The work relates to Gao Jian's studies on cisplatin-resistance in OSCC. She induced cisplatin resistance in previously sensitive cell lines to examine the intracellular mechanisms for drug resistance. During this work she discovered that the Amphiregulin mRNA levels varied in a cyclic manner.

Although cyclic mRNA fluctuation is the main topic, the thesis will include an introduction of basic elements which relate to the treatment of OSCC – beginning with the chemotherapeutic drug *cisplatin*; a platinum-based drug also known as *cis-diamminedichloroplatinum (II)*. [4]

1.2 Cisplatin – the chemotherapeutic miracle?

Cisplatin was first used to treat cancer in 1971. [4, 5] Today, it is still the drug of choice when treating a wide variety of malignant solid tumors, primarily of the testes, ovaries, urine bladder, esophagus and head and neck squamous cell carcinoma (HNSCC). [5, 6] It is one of the most effective broad-spectrum anticancer drugs [7] and can be used either alone or in combination with other drugs and adjuvant treatments. [5] For HNSCC, it is often combined with radiotherapy. [8]

The mechanism of cisplatin is mainly through its platinum contents, which form covalent bonds to DNA purine bases, creating DNA adducts. [4] The DNA adducts cause structural changes of the DNA – e.g. through bending and unwinding – which leads to inhibition of DNA replication and RNA transcription. This ultimately leads to cellular apoptosis. [4, 9] The precise cellular mechanisms of the platinum-DNA adducts' induction of cell death are not yet fully understood. [9]

1.3 Treatment resistance

The most prevalent clinical problem of cisplatin is the development of tumor *resistance*, a feature which becomes evident when cancer cells do not undergo apoptosis at clinically relevant drug concentrations. [9] The mechanisms of cisplatin-resistance are divided into two main categories: "Intrinsic resistance", which describes resistance in previously untreated tumor cells and "acquired resistance", in which repeated exposure to cisplatin triggers resistance. [5]

The potency of cisplatin is complex due to its many routes of entry and multiple cellular pathways, therefore allowing the development of cisplatin resistance to occur through equally complex changes in gene expression. [7] Many studies have been conducted to better understand the mechanisms of drug resistance, in hope of eventually increasing cisplatin efficiency by combining it with resistance modulators. [4]

1.4 Studying the molecular pattern of resistance

Squamous cell carcinomas (SCC) derive from keratinocytes. Amphiregulin (AREG) is a growth factor in the epidermal growth factor (EGF) family and is an established oncogene in epithelial malignancies. [10] In this paper, we have studied the levels of AREG in different cisplatin resistant oral cancer cell lines, but before looking at the methods and results; let us begin by better understanding the target molecule, *amphiregulin*.

1.5 The answer might be in amphiregulin

Several recent publications have identified AREG as a key factor for cisplatin resistance in human breast cancer. [11, 12] Eckstein et al. discovered a significant correlation suggesting that breast cancer cells used AREG as a survival signal upon exposure to cisplatin. [11] However, this was not observed for lung cancer cells – samples did not show a connection between cisplatin resistance and amphiregulin expression, despite elevated amphiregulin-levels. At the very end they state:

> **"[…]it is necessary to systematically investigate different tumor types to determine the role of amphiregulin for cisplatin resistance in other malignant tumors besides breast cancer."** (Eckstein et al.) [11]

There is a lot of interest in how AREG may be involved in the development of cisplatin resistance. It has been suggested that AREG-expression has a tumor promoting capability

for oral squamous cell carcinomas (OSCC). [13] Many researchers believe that this gene may be a target for future resistance modulating chemotherapeutic drugs!

1.6 Amphiregulin

Amphiregulin (AREG) is a protein, originally identified in the human breast carcinoma cell line MCF-7. [15] AREG is an 84-amino acid glycoprotein which, in its mature form, has a three-looped secondary structure. [10] This mature form is due to six cysteines, in the polypeptide chain, which constitute three disulfide linkages conserved in all the EGF family ligands. [16] It is a ligand which binds and activates the epidermal growth factor receptor (EGFR). [10, 12]

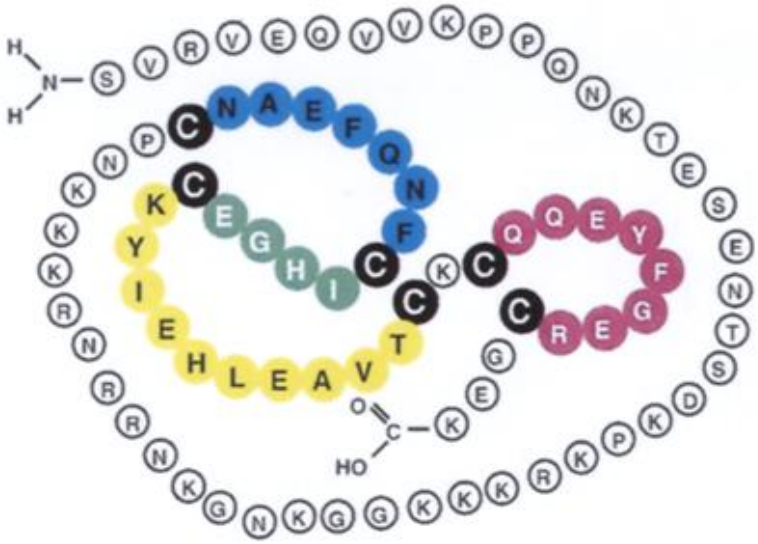


Figure 1: The secondary structure of human amphiregulin. The six black C's represent cysteines which join together, forming three disulfide links and three loops. This structure is conserved in other EGF family ligands. [14]

The name – **amphi**regulin – is given due to this growth factor's ability to act stimulatory on certain cells and inhibitory on others. [12] AREG was previously recognized for its ability to inhibit growth of human carcinoma cells in culture and stimulate proliferation of human fibroblasts. [15] In newer literature, it has been recognized as an oncogenic factor. [10, 13] Its overexpression has been identified in HNSCC and in a wide variety of other human cancer tissues. [10, 16] The study which first identified this phenomenon concludes that there is a significant auto- and cross-induction among the different EGFR ligands, suggesting that it is not only AREG that could have tumor-promoting abilities. [16]

1.7 The EGFR family

The EGFR belongs to a group of receptor tyrosine kinases known as the ErbB or human epidermoid receptor (HER) family. [10, 12] This receptor family contains four members: EGFR/ErbB1/HER1, ErbB2/HER2/Neu, ErbB3/HER3 and ErbB4/HER4. [10, 17] The ligands which bind to the ErbB receptor family are: amphiregulin (AREG/AR), epidermal growth factor (EGF), transforming growth factor alpha (TGF-α), heparin-binding EGF-like ligand (HB-EGF), betacellulin (BTC), epiregulin (EREG), epigen (EPGN) and neuregulin (NRG) family members. [11] All these ligands belong to the EGF family. [11] The mature form of AREG shares a structural homology with other ligands in the EGF family and all the ligands share at least 28 percent sequence identity. [10, 16]

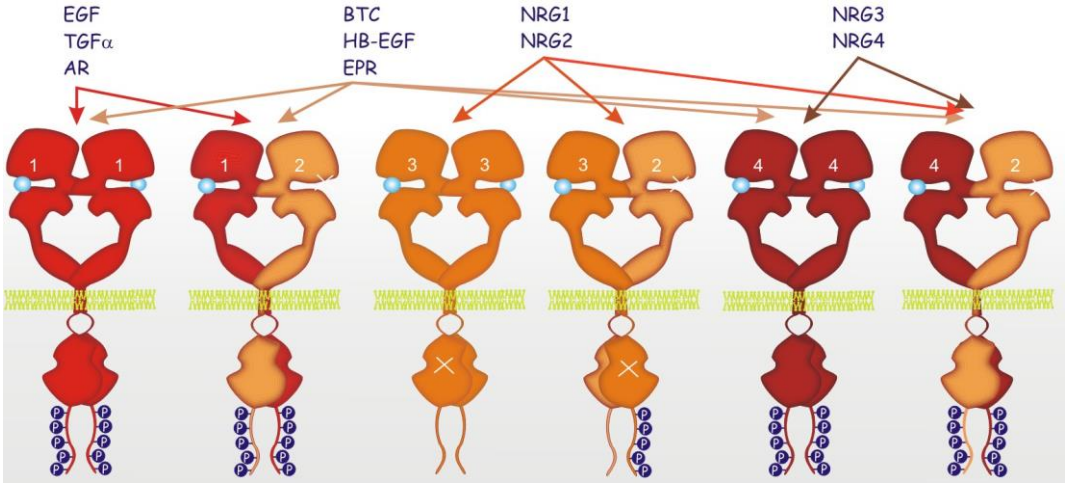


Figure 2: The ErbB-receptor family and ligands. After activation by a ligand, this is the resulting receptors after dimerization of the individual receptor tyrosine kinases. The conformational changes which occur along with dimerization activate different intracellular pathways depending on the many homo- and hetero-dimerizations. [18]

The receptors in the ErbB-family share a structural homology. [10] They are single-pass, transmembrane receptor tyrosine kinases (RTKs). [19] The receptors are activated upon ligand-binding, which induces conformational changes in the extracellular domain. This facilitates the receptor's homo- or hetero-dimerization with other family members [20], further resulting in the initiation of intracellular signaling cascades. Each EGF family ligand stabilizes different dimers and each dimer recruits different combinations of signaling molecules. [21]

1.8 The discovery of amphiregulin-fluctuations

The complexity of the amphiregulin (AREG) molecule is what inspires scientists to study its mechanism – a fact which also applies for this thesis. When we first began studying cisplatin resistant and cisplatin sensitive cell lines, the aim was to explore whether there was a significant correlation between AREG-levels and cisplatin resistance. Upon working with these cells, it was discovered that the AREG-levels fluctuated with a seemingly consistent pattern over time. Whether these fluctuations were accurate or simply coincidental is the focus of this thesis.

Before delving into the details of the laboratory work in parts 3 and 4; the next section will give a brief familiarization with certain expressions and include a bit of relevant theory.

Part 2: Genetics and background

2.1 Gene expression

The DNA codes our hereditary material, but only a portion of the DNA is actually expressed. The term 'gene expression' comprises the process of converting our DNA material into a product which has an effect on a cell or organism. [22] Protein synthesis is a major part of gene expression and to induce protein synthesis the messenger RNA (mRNA) needs to serve as a link. [23, 24] The mRNA carries the genetic information from the nucleus to the ribosomes in the cytoplasm, where it serves as the substrate for translation. [25]

2.2 mRNA: Transcription and structure

The basic layout of transcription contains the three steps: initiation, elongation and termination. During the *initiation* step, the enzyme RNA polymerase II (RNAPII) binds a specific sequence – the "promoter" – at the 5' end (also termed "upstreams") of a DNA template. After synthesizing approximately 10 nucleotides, the *elongation* process begins; the RNAPII enzyme synthesizes polynucleotides as encoded by the DNA, until it reaches a "termination signal". In the *termination* step, RNAPII releases both the DNA template and RNA product. [22, 26]

The mRNA molecule, just like DNA, has a 5' and 3' ends. At the 5' end there is a *cap* consisting of guanine (G) nucleotides with a methyl group. At the 3' end, the mRNA has a long link of several hundred adenine (A) nucleotides, commonly known as the *poly(A) tail*. The mRNA is transcribed by the RNAPII within the cell nucleus and the cap and tail modifications occur before the transcripts are exported to the cytoplasm, and they contribute in the identification of RNA molecules as mRNA. Both ends need to be present before protein synthesis can be initiated. [22]

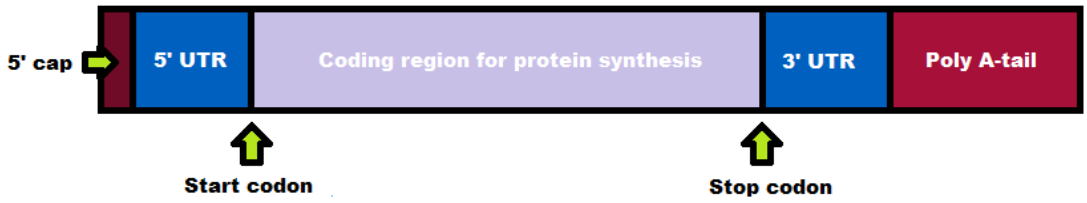


Figure 3: The segments of a mature mRNA consist of a guanine cap at the 5' end, an untranslated region (UTR), a coding region for protein synthesis (the region varies depending on transcribed gene), a 3' UTR and, finally, a poly(A) tail. The start and stop codons flag the starting and ending points for the translational machinery. [22]

2.3 mRNA regulation

The mRNA-conversion from a pre-mRNA into a functional mRNA is an important step in gene expression. Traditionally, this process involves 5' capping, intron splicing and polyadenylation at the 3' end. [27, 28] But newer research has discovered novel co- and post-transcriptional regulatory mechanisms, which also coordinate gene expression.

Pre-mRNA to mature mRNA

Capping is an mRNA modification which adds a guanosine cap. This process depends on three enzymes working in consecutive order: RNA triphosphatase, RNA guanylyltransferase and RNA-(guanine-7)methyltransferase. The cap functions by preventing degradation, while stimulating splicing and polyadenylation. [28, 29] *Splicing* alters transcription by removing introns and ligating exons in different patterns, allowing the same transcript to encode several different proteins. [29] *Polyadenylation* occurs when the RNAPII reaches the 3' end of the coding region, this triggers a cleaving at the poly(A) site (AAUAAA) and leads to the addition of a poly(A) tail. [30] The poly(A) tail controls mRNA stability and translation by controlling the length of the of the 3' UTR – the site for further mRNA regulation. [27]

Co-transcriptional regulation

RNA folding is an important for regulatory step and the folding can occur both co- and post-transcriptionally. Continuing research and progress exists for this topic. Three (of many) co-transcriptional steps, with the ability to incur co-transcriptional influence over RNA conformation, are listed below.

- **Transcription speed**. The transcription of the 5' end can take place up to several seconds prior to the 3' region. This dictates the folding, as base coupling only occurs between previously synthesized RNA portions. [26]
- **Transcription pausing**. Several factors dictate the nature of transcriptional pausing. A stop of the RNAPII can last a couple milliseconds up to seconds and influences RNA folding through the length of this pause. [26]
- **Co-transcriptional interactions**. RNA binding proteins (RBPs) or RNA helicases interact with nascent RNA to *prevent* base pairing with the selected site or to *facilitate* interaction. [26]

Post-transcriptional regulation

In post-transcriptional regulation of mature mRNA, it is mainly the UTRs targeted by *trans*-factors – such as RNA-binding proteins (RBPs) or microRNAs (miRNAs) – which steer the regulatory events. [27] The UTRs make up the *cis*-elements which interact with cellular *trans*-factors, together creating large, interacting macromolecules. [31] Examples of the activity of *cis*-acting and *trans*-acting factors are listed in **table 1.**

***Cis*-acting elements**	***Trans*-acting elements**
Poly(A) binding protein (PABPC1): Is deposited on the poly(A) tail and participates in translation initiation and termination	**5' UTRs:** Contains regulatory elements such as IRES (Internal Ribosome Entry Site) and IRE (Iron Responsive Elements) involved in translation regulation. Also required for binding decapping enzymes for inducing mRNA degradation.
CCR4-NOT4: Promotes transcription elongation and regulates mRNA decay.	**Coding sequence:** Regions with many functions, for exmaple miRNA-binding sites and exon-exon junctions (EEJ), which guide splicing.

Table 1: This table contains examples of *cis*- and *trans*-acting elements in mRNA regulation. [27]

Although RBPs were previously considered the "primary mRNA regulators", more recent studies have shifted this role to microRNA (miRNA). [31] The miRNA are among the most abundant regulatory genes in vertebrates. [3, 25] In humans, miRNA regulate more than 60 percent of the protein-coding genes. [31] The miRNAs will be discussed in greater detail in the 'Discussion'-section towards the end.

2.4 mRNA quantification

Cellular functions are expressed through varied levels and patterns of mRNA. The ability to quantitate mRNA concentration and to understand the transcription of individual genes has become exceedingly useful in many branches of molecular science, for example to study drug resistance markers in tumor cells, such as AREG in cisplatin resistant cells. [32]

The four methods commonly used for quantification of transcriptions are:

- Northern blotting
- *In situ* hybridization
- RNase protection assays
- Reverse transcription polymerase chain reaction (RT-PCR)

Each method has its own strength, but the common limitation of the first three techniques is a relatively low sensitivity. The reverse transcription polymerase chain reaction is acknowledged as the most sensitive method. [32] Its great strength is its ability to amplify and analyze even the smallest amounts of DNA in cell samples. But due to the mRNA's inherent instability, it must be reversed transcribed into complementary DNA (cDNA) by retrovirus' reverse transcriptase, prior to PCR and DNA polymerase activity. [32, 33]

2.5 Polymerase chain reaction

Polymerase chain reaction (PCR) is a method for DNA amplification developed by Kary Mullis in the late 1980s. [34] With this method, any DNA template can be copied through repeated sessions of DNA replication. By adding the desired DNA primers, it will pair with the existing DNA strands and thereby guide the DNA polymerase to copy the following sections. [22] The amount of DNA molecules will increase exponentially as the replication proceeds, therefore, only a small amount of sample DNA is needed to create a much larger DNA pool. [34]

The *DNA primers* are designed and synthesized oligonucleotides containing 15-20 bases of DNA. To start a PCR reaction, the operator must know the beginning and end sequence of their target DNA. [22] The primers provide the 3' end for the DNA polymerase to start replicating and two primers will initiate a parallel occurring DNA synthesis, in opposite directions.

The replication will begin by heating the template to a high temperature (e.g. 95 degrees), to separate the double-stranded DNA. Then the temperature is lowered, a sufficiently lowered temperature will allow the primers to bind and hybridize with their complementary sequences. A somewhat higher temperature will cause elongation, and then the process of heating, separating and extending will begin anew. [34]

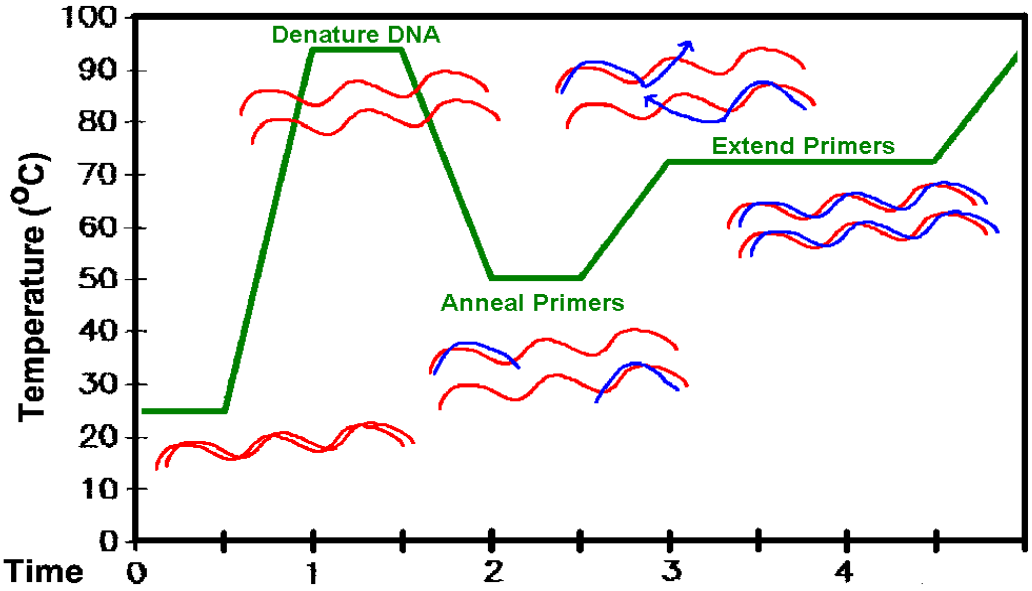


Figure 4: From the beginning, the reaction mix contains both **DNA** and **primers**. In the first phase (*denaturing*), the reaction is heated to a temperature of 95°C. The **dsDNA** separates. This leaves two strands of **ssDNA**. The reaction is then cooled to ~50°C, entering its second phase (*annealing*). This temperature allows the **primers** to bind complementary **ssDNA**. In the third phase (*extension*), the reaction is reheated to 72°C and the **primed ssDNA** will replicate. With the end of replication, comes the end of the first cycle. The cycles are repeated many times, until the desired sample amount is achieved. [35]

PCR developed for the purpose of determining the amount of specific mRNA or DNA is often referred to as *quantitative PCR* or *real-time PCR* (qPCR).

2.6 Real-time PCR

When qPCR is used for mRNA quantification, the mRNA needs to be reverse transcribed (RT). The two methods, reverse transcription and qPCR, are often coupled together and the combination has become an important tool within gene expression analysis. [36] The joined practice is commonly known as reverse transcription quantitative polymerase chain reaction (RT-qPCR) or real-time reverse transcription PCR (real-time RT-PCR) and has become the "gold standard" for absolute and relative quantification of mRNA levels. [33, 37, 38]

To accurately quantify cDNA, a reaction mix with primers and a fluorescent reporter dye is necessary. Through the entire amplification reaction, the dye will continuously bind to the PCR products. The computer registers the increase in fluorescent signal as the signal reaches the threshold for detectability. An automatic calculation estimates the sample's original concentration. This is possible because the fluorescence is proportional to the amount of DNA product. [32, 39]

Part 3: **Materials and methods**

3.1 Experimental procedures

3.1.1 Cell culture

The two human OSCC cell lines PE/CA-PJ34 clone C12 (male, 60 years) from the oral cavity and PE/CA-PJ49 clone E10 (male, 55 years) established from tongue tissue, were cultured in standard medium (IMDM, Sigma-Aldrich, St. Louis, MO, USA), supplemented with 10% fetal bovine serum (FBS) (Cambrex, Verviers, Belgium), 2 mM L-glutamine, and 1% penicillin-streptomycin (PS) (Cambrex) at 37°C and 5% CO_2. C12 is cisplatin sensitive, E10 is cisplatin resistant.

Cells were seeded at a density of 2 x 10^5 cells per well in 12-well microtiter plates (nunc, Wiesbaden-Biebrich, Germany), that were at 80% confluency, in 2 µl IMEM with 10% FBS per well. Cells were harvested at 10 a.m, at time intervals of 15 minutes, 30 minutes or 2 hours (specified in later in the text, for the individual experiments). The cell-culture medium was completely aspirated and washed twice by PBS, then added 350 µl Buffer RLT (RNeasy, Qiagen) in each well, pipetted a few seconds to get cells fully disrupted and afterwards transferred to 2 ml tubes and kept on ice. At this point, cell samples were ready for RNA isolation.

3.1.2 mRNA isolation

The cell precipitate was removed from the freezer upon mRNA extraction. Samples were stored at room temperature until liquid form. Under an RNase-free ventilated drain, the samples were added with 350 µl 70% ethanol. The entire content of 700 µl was then transferred to the RNeasy spin column (Qiagen) and centrifuged 1 minute (temperature 24°C, speed 130 rotations per minute). Each sample was first washed with 700 µl RW1 buffer, the precipitate was removed and the columns were centrifuged for 1 minute. Then we added 500 µl RPE wash buffer, removed the precipitate and centrifuged for 1 minute. This process was repeated, and then centrifuged for 2 minutes. Afterwards, the top of the RNeasy spin tube was moved to a new 2 ml collection tube and re-centrifuged for 2 minutes. Precipitate was moved to a lidded 1,5 ml collection tube. Depending on the cell amount in the original samples, each tube was added with water (30-50 µl) and let rest for 1 minute, then centrifuged for 1 minute. The precipitates were moved to new tubes.

RNA samples are extremely susceptible to being destroyed by ribonucleases, so when working with RNA, gloves were used at all times. Due to low tolerance of high temperatures, the samples were kept in a freezer at minus 80^{o}C and put on a cooling element when in use, according to recommendations. [40]

3.1.3 Nucleic acid concentration

Both the RNA quality and quantity needs to be assessed, without exact values it cannot be further utilized. Sample nucleic acid concentration was measured by the NanoDrop 2000c Spectrophotometer. The settings were adjusted for measuring RNA. The NanoDrop was then reset by calibrating the machinery to the contents of 1 µl RNase free water, set at "blank", then cleaning the dispenser and adding the RNA samples. The nucleic acid concentrations were used to calculate the necessary µl sample needed to proceed with the reverse transcription, and each sample were also checked for their A260/A280-value, each ensured to be within the purity ratio of 1,8 and 2,2.

3.1.4 Reverse transcription

Reverse Transcriptase Core Kit (Eurogenetec) was utilized for the reverse transcription procedure. The instructions under "Procedure for Two step qRT-PCR reaction" were followed. The 10x reaction buffer, 25mM $MgCl_2$, 2.5 mM dNTP and random nonamer were removed from the freezer and gently warmed at a temperature of 37°C, until the contents were liquefied. The reagents were added to a reaction vial (Eppendorf DNA LoBind Tube 1,5 ml) and mixed. Then, the RNAse inhibitor and EuroScript RT were added. The vial was put on ice, as RNA was added. RNA was calculated for total RNA concentration <200ng. The RT Reaction Mix was distributed into new tubes (Stratagene Optical Tube, 8x Strip) with the sample mRNA, then diluted with RNase free water, as needed, and finally lidded (with Agilent Technologies Optical Cap, 8x Strip). The finished sample was loaded into a preheated GeneAmp® PCR system 9700, for 45 minutes (10min at 25°C, 30min at 48°C, 5min at 95°C, then cooled down at 4°C until the operator manually terminates the program). The program settings were typed according to tube's ml contents. The finished sample was stored in a freezer until the cDNA is needed.

3.1.5 Quantification by real-time PCR

Real-time PCR was performed using RealMasterMix with added SYBR Green (Eppendorf); 1,0 ml of the 2,5x RealMasterMix added 125 µl of 20x SYBR Solution. The reaction mix was

calculated according to the manufacturer's description. Forward primers and reverse primers (Sigma-Aldrich, Eurogentec) were added, for target gene and housekeeping gene (for exact sequences and manufacturer, see **table 2**). Sample cDNA was diluted with RNase free water, with a 1:3 ratio (sample:water). Trays of 96 wells were loaded with 8,4 µl reaction mix and 6,6 µl sample cDNA. At least two parallel wells were loaded with sample and the normalizer, respectively. In each tray, the last two wells in sample and the normalizer group were added 8,4 µl RNase free water, instead of reaction mix to ensure controls with no Ct-value. The completed tray is centrifuged with Megafuge 1,0R's "quick run" function up to a speed of 2000 rcf. The finished tray is then placed within the Mx3005P for quantification. Data was acquired through the MxPro QPCR Computer Software.

Gene		Forward primer (Fw)	Reverse primer (Rv)
TBP	TATA-box binding protein	5'-CGTGGCTCTCTTATCCTCATGA-3'	5'-GCCCGAAACGCCGAATATA-3'
HPRT1	Hypoxanthine phosphoribosyl-transferase 1	5'-TTGACACTGGCAAAACAATGC-3'	5'-GCTTGCGACCTTGACCATCT-3'
AREG	Amphiregulin	5'-GCTCAGGCCATTATGCTGCTG-3'	5'-ACTCACAGGGGAAATCTCACTCC-3'
EGFR	Epidermal Growth Factor Receptor	5'-GTGGCATTTAGGGGTGACTC-3'	5'-CAGAATATCCAGTTCCTGTGGA-3'
EGF	Epidermal Growth Factor	5'-GGTGGTGAAGTTGATCTAAAG-3'	5'-TAGCATGTGTTGAGATTCTG-3'

Table 2: The primer sequences. , TBP, HRPT1, AREG, EGFR and EGF were purchased from the manufacturer Sigma-Aldrich. EGFR is from Eurogentec.

There are many fluorescence-based technologies, in our lab we are experienced with using SYBR® Green, a fluorescent dsDNA binding dye. [38] When the PCR cycle amplifies the samples, the fluorescence will reach a threshold value of background fluorescence – this parameter is known as the *threshold cycle* (Ct) or *crossing point* (Cp). The larger the sample is at starting point, the lower the Ct-value. Based on the fluorescence and amplification product, calculations can be made for an accurate quantification of sample molecules. [39]

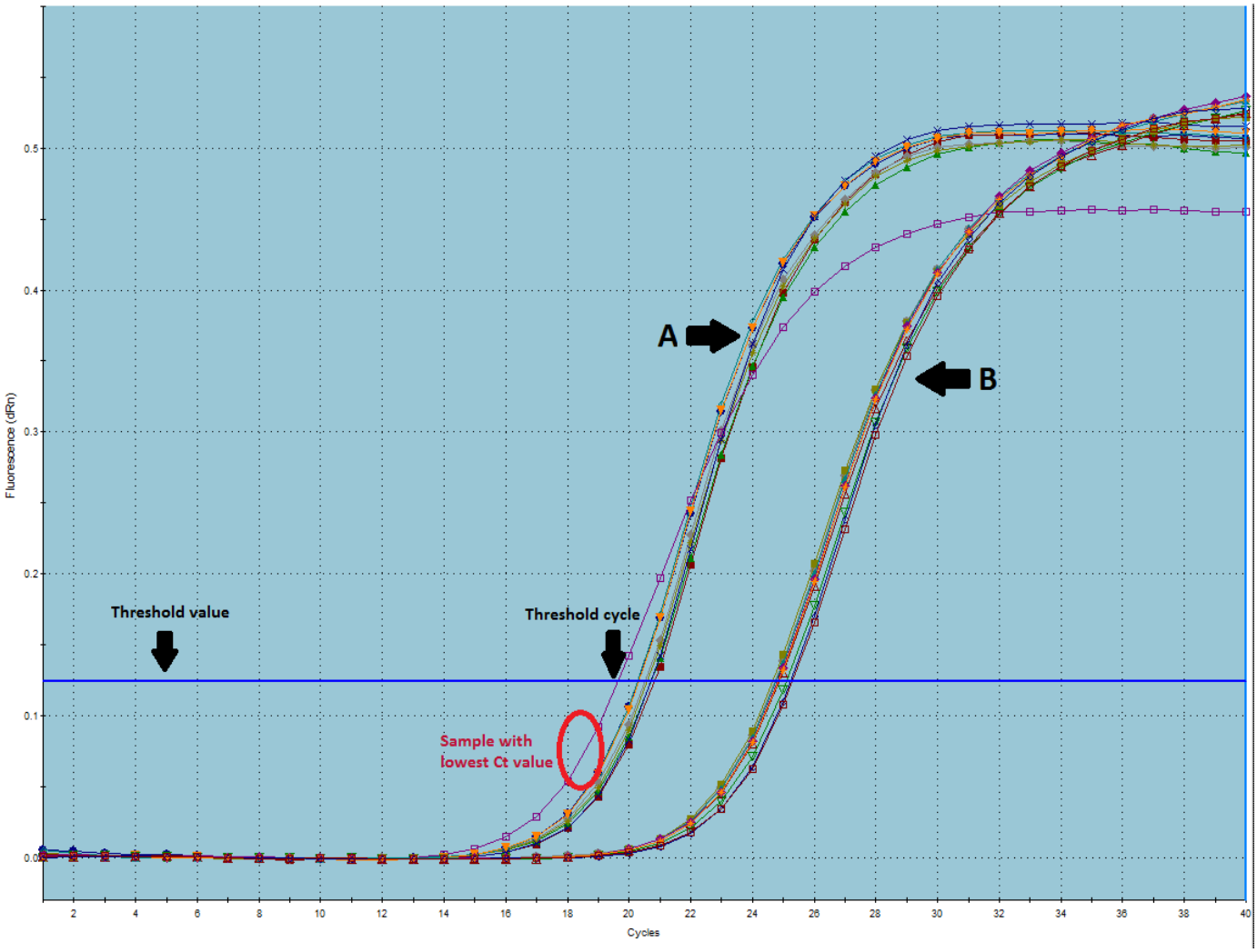


Figure 5: The amplification plot from qPCR (Mx3005P) as displayed in the MxPro QPCR Software. The **blue line** represents the threshold level; the lower limit for detection of the amplification products. When the graph crosses this mark, this results in a Ct-value or "threshold cycle", which corresponds to the number on X-axis. The **red ring** marks the sample with the lowest Ct, this is the sample with the highest sample concentration (this corresponds to peaks at 1,5h in **figure 11**). **(A)** The cluster of lines represents the target gene. **(B)** Cluster of lines shows reference genes amplification pattern.

All	1	2	3	4	5	6	7	8	9	10	11	12
A	Calibrator 23.26	Calibrator 23.32	Calibrator 23.20	Unknown 24.20	Unknown 24.23	Unknown 24.16	Unknown 23.29	Unknown 23.53	Unknown 23.36	Unknown 22.74	Unknown 22.79	Unknown 22.79
B	Unknown 22.75	Unknown 22.86	Unknown 22.82	Unknown 22.47	Unknown 22.43	Unknown 22.45	Unknown 23.22	Unknown 23.16	Unknown 23.20	Unknown 22.79	Unknown 22.68	Unknown 22.61
C	Unknown 22.64	Unknown 22.77	Unknown 22.70	Unknown 21.12	Unknown 20.96	Unknown 20.99	Unknown 20.86	Unknown 21.96	Unknown 20.86	Unknown 22.33	Unknown 22.25	Unknown 22.26
D	Unknown 21.33	Unknown 21.40	Unknown 21.33	Unknown 21.81	Unknown 21.69	Unknown 21.67	Unknown 21.30	Unknown 21.28	Unknown 22.40	Unknown 22.34	Unknown 21.55	Unknown 21.46
E	Calibrator 30.26	Calibrator 29.85	Calibrator 30.14	Unknown 30.89	Unknown 31.02	Unknown 30.96	Unknown 30.36	Unknown 30.79	Unknown 30.20	Unknown 29.58	Unknown 29.54	Unknown 29.46
F	Unknown 29.66	Unknown 29.71	Unknown 30.03	Unknown 29.50	Unknown 29.25	Unknown 29.32	Unknown 30.65	Unknown 30.16	Unknown 30.56	Unknown 29.84	Unknown 29.63	Unknown 30.20
G	Unknown 29.75	Unknown 29.98	Unknown 30.00	Unknown 27.68	Unknown 27.66	Unknown 27.51	Unknown 27.33	Unknown 27.35	Unknown 27.31	Unknown 29.53	Unknown 29.23	Unknown 29.59
H	Unknown 27.47	Unknown 27.43	Unknown 27.45	Unknown 28.28	Unknown 28.20	Unknown 28.04	Unknown 27.82	Unknown 27.69	Unknown 28.54	Unknown 28.50	Unknown 28.15	Unknown 28.08

Figure 6: The 96-well tray after a completed PCR as displayed in the MxPro QPCR Software. The blue numbers are the Ct-values of the added samples. Parallel wells (two or three which share the same number and letter, e.g. 1A) should not show values which diverge more than +/- 0,8 relative to each other. The average Ct-values of the parallel wells were used for the data presentation in part 4.

3.2 Methodological considerations

3.2.1 RNA isolation

RNA preparation and isolation methods may vary greatly. In this research, the RNA isolation method was a *filter based* or "spin basket" type. This uses a membrane - a silica membrane in the RNeasy spin column – which binds the desired product, while other organic contents pass through and become filtered out by centrifugation and then discarded. [41]

Other extraction methods include:

- *Organic extraction:* The chosen sample is combined with a solution which, after centrifugation, separates the sample in three phases. The first upper layer is the RNA. [41]

- *Magnetic particles:* By adding small, magnetic particles which target sample particles will make it possible to extract molecules of interest by using a magnetic field. [41]

- *Direct lysis:* Simple extraction method, applied by adding a lysis buffer which stabilizes the nucleic acids. [41]

All the methods have their pros and cons. Sadly, there is no established "standard" method for RNA isolation. The spin basket isolation method is regarded as an overall good method for RNA purification, but as it yielded a relatively low 260/230 purity number it may be indicative of choosing a spin basket technology other than Qiagen's RNeasy kit for future research. Fortunately, this number has no impact on the qPCR results. On the other hand, sample 260/280 purity were within the desired range. An explanation of these values and their importance can be found below.

3.2.2 Nucleic acid purity

The assessment of nucleic acid purity preliminary to qPCR is important for reliable, final results. It is to control whether the purification of sample contaminants is adequate, and the evaluation is completed by studying the 260/280 and 260/230 ratio of sample RNA. These numbers were established with the NanoDrop spectrophotometer, a device which measures the absorption of light with wavelengths 230, 260 and 280 nanometers (nm). Products which absorb the different wavelengths:

- 280nm - absorption of proteins (specifically aromatic amino acids) [42]
- 260nm - absorption of nucleic acids (DNA & RNA) [42]
- 230nm - absorption of organic contaminants and solvents [42]

All nucleotides, RNA, ssDNA (single-stranded DNA) and dsDNA (double-stranded DNA) will absorb waves at the 260 nm wavelength and increase the total absorbance of the sample. [43] In summary, the NanoDrop assesses both the *quantity* of the RNA (nucleic acid concentration in ng/µl) and the *quality*. The quality is described by the two aforementioned ratios, the separate numbers convey the following information:

- 260/280

The RNA quality is determined by analysis of the 260/280 ratio, a number which expresses the sample's purity; its absence of proteins, DNA contamination and inhibitors. [39] A ratio

of approximately 2,0 is generally accepted as pure RNA. As the ratio decreases, the purity also decreases, because the protein contamination rises. [42] In our selection, the samples have abided by the recommendation of a 260/280 ratio ~2, samples with a maximum deviation of 0,2 in a positive or negative direction (+/- 0,2) were included in the experiments.

- 260/230

Secondary to the 260/280 ratio, there is the 260/230 ratio. Values for the 260/230 ratio are expected to fall within the range of 2,0-2,2. If the ratio is lower, this indicates that there may be contaminants that absorb wavelengths at 230 nm. [44] The 260/230 ratio has had no value in the selection or disposal of samples, as this specific ratio is insignificant in RT-qPCR.

3.2.3 RT primers

When choosing primers for the reverse transcription and cDNA synthesis, it has been debated whether random nonamers or poly-dT would serve as the best primer. It is implied that, as the poly-dT primer begins by binding samples at the complementary poly(A) tail and needs to transcribe the mRNA's entire length, a poly-dT primer will result in a lesser but more accurate amount of cDNA product, when compared to transcription by random nonamers. This is additionally explained by speculating that random nonamers will bind randomly to all the RNA and facilitate transcription of both the desired RNA product, as well as the partially degraded RNA. [45] Because of this ambiguity, experiments with the use of poly-dT and random nonamer primers were conducted. No obvious discrepancy was observed by the two parallel methods.

3.2.4 Quantification method

As previously established, qPCR is a great and common choice for quantification of DNA and RNA. Nevertheless, there are certain elements in the procedures from the RNA extraction to the completed PCR which can create variations and instabilities in the results. [47] There are two ways for DNA quantification in RT-qPCR – absolute and relative quantification. We have used the latter, as it is widely used and sufficient for our purpose as explained by Michael W. Pfaffl:

> **"Relative quantification is easier to perform than absolute quantification [...]. It is based on the expression levels of a target gene versus a housekeeping gene (reference gene or control gene) and in theory is adequate for most purposes to investigate physiological changes in gene expression levels."** [46]

This type of data normalization relies on a good and stable reference gene for correct results. [48]

3.2.5 Housekeeping genes for qPCR data normalization

The use of housekeeping genes (HKGs), or endogenous reference genes, is widely regarded as a suitable method for data normalization in RT-qPCR. The HKGs are *endogenous* as they already exist in the sample and function in the coding of the most fundamental cellular activities and processes. HKGs are necessary for the homeostasis of the cell and should normally be expressed at constant levels. [47, 49] By comparing the Ct of the target gene sample with the Ct of the HKG, the gene expression can be normalized. [50]

Although these genes are assumed to be relatively stable, there is increasing evidence that many of the HKGs are not as resistant to environmental changes as it was previously thought. [47] A review by Bär et al. points out that different types of HKGs were appropriate for the study of adult keratinocytes, while not every one were appropriate for studying juvenile keratinocytes. [51] In another review, by Jacob et al., it was noted that GADPH was among the least stably expressed reference genes, despite being the most frequently used, as different disease stages could alter its gene expression. [38]

To optimize the normalization, the reference genes should embody certain qualities:

- Expression at constant levels in different tissues. [33]
- Consistency through all environmental and experimental conditions. [47]
- Expression at similar levels as the studied RNA. [33]
- Should undergo all steps of the reaction with the same kinetics as the target gene. [33]

Accurate normalization of gene expression is an absolute prerequisite for precise quantification of target nucleic acid, which is why the study of HKG-reliability is such an important part of qPCR. In a study by Radonić et al., RT-qPCR was used to measure mRNA of 13 HKGs in 16 different human tissues, with the aim of determining which were the most stable. [52]

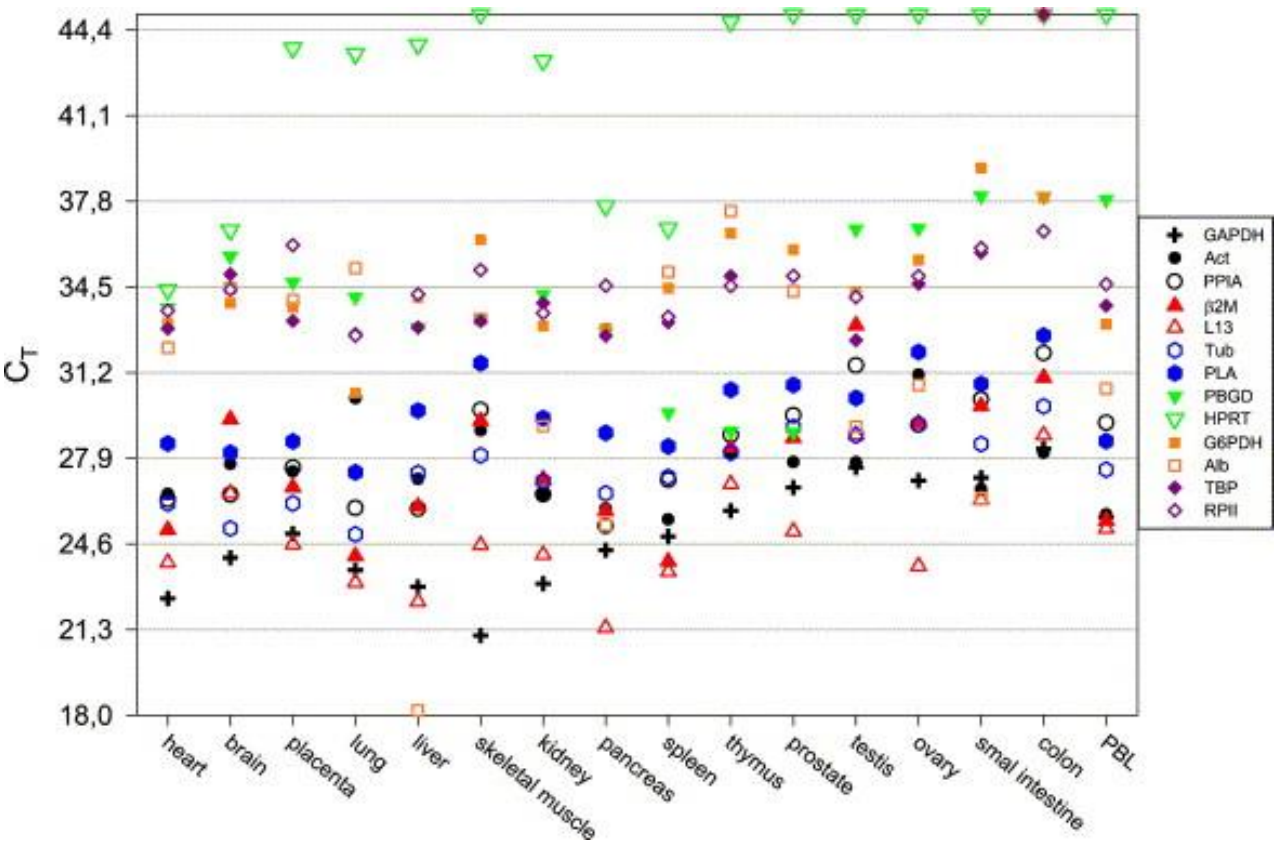


Figure 7: The RNA transcription levels of 13 HKGs in different tissue samples, showing how the levels vary in different tissues. Some samples show greater tissue variations than others. Ct ≥ 45 is not depicted. [52]

The results among the 13 HKGs were varying. None of the HKGs showed stable expression levels in all the tissue samples; each expression level shifted from one tissue to another, but some samples showed a much greater variation.

To estimate the extent of diversity, the range between lowest transcription level (high Ct) and highest transcription level (low Ct), was depicted in percentiles. The gene with the lowest range should suggest a relatively constant transcription level in the 16 tissue samples and therefore trustworthy. [52]

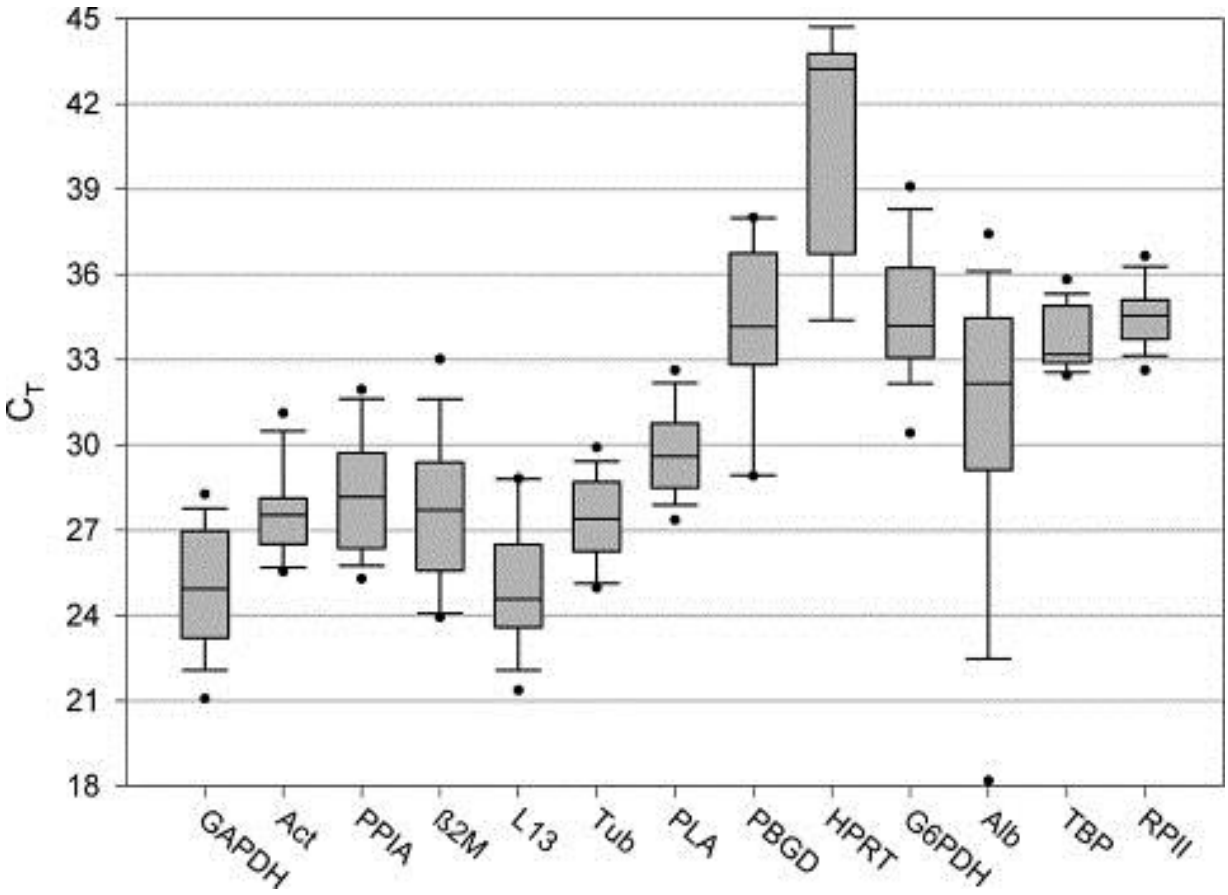


Figure 8: The ranges between lowest and highest Ct-values are depicted. Grey boxes mark the 50% percentile, with a black line within representing the median. Vertical whiskers indicate the 80% percentiles. Dots are outlying results. TBP, RPII and Tub showed the lowest ranges, and are therefore assumed to be the most stable. On the opposite end of the spectrum; Alb, HPRT and PBGD displayed the largest ranges. [52]

The results argue that TBP is most stably expressed HKGs among the chosen 13, while HPRT is among the least stable. A publication by Minner and Poumay also suggest the use of TBP, but preferably together with two other HKGs. [53]

In the results, which will be presented shortly, we have followed the recommendation of using multiple HKGs to minimize errors. [52, 54] The two reference genes used for qPCR were TBP and HPRT, chosen primarily due to availability. This should be taken into consideration if the experiments were to be repeated, as there could be HKGs better suited for the study of OSCC, than the two in our selection.

3.3 Experimental background

During the introduction it was already mentioned how the very first experiment relating to mRNA fluctuations was actually executed by one of my supervisors, Ph.D-candidate Jian Gao. This experiment studied cell samples which were handled and harvested with 2 hour intervals. The PCR results showed fluctuations in the amphiregulin-levels. This sparked interest but it also inspired questions.

- Are the fluctuations cyclic occurrences or random peaks?
- Are the 2 hour intervals depicting an accurate rendition of the mRNA cycles?
- Is the experiment repeatable?

Part 4: **Results**

4.1 Data presentation

My work has primarily involved mRNA extraction, reverse transcription and quantitative PCR runs. Different cell samples have been studied and each sample was subjected to several reverse transcriptions and PCR runs. The charts which will be presented in this thesis are chosen from PCR results which best represent the many trials – a sort of "median" of each sample. The total number of completed PCR runs is summarized in **table 3**.

The composition of experiments – choice of primers and housekeeping genes – were decided in a dynamic manner, meaning that as the work was conducted, questions arose and this became the guide for choosing these variables. The original intent was to study the amphiregulin fluctuations – to verify or deny the existence of periodical peaks.

Total number of PCR runs = 18	
Divided by sample gene primer:	
AREG	12
EGFR	1
EGF	2
HRPT	3
Divided by RT-primer	
RN	16
Poly-dT	2
Divided by HKGs:	
TBP	14
HRPT	4

Table 3: All the 18 PCR runs, subdivided by the use of DNA primer, reverse transcription primer and HKGs.

Each experiment studied cells harvested with a defined interval in time, throughout a defined period of time. Then the Ct-values of the HKG are subtracted from Ct of sample gene, producing in the **ΔCt**value. The **ΔCt** of the first sample (defined as the 'calibrator' or 0h) is subtracted from the **ΔCt** of another sample, e.g 0,5h, and results in the value **ΔΔCt**. The negative value, **-ΔΔCt**, is added as an exponent of 2. The resulting number marks a coordinate in a graph. Calculations are summarized in **figure 9**. The $2^{-\Delta\Delta Ct}$ method is used, as recommended by Jensen, for creating the data presented in the following plots and charts [33], unless other values are specified.

Ct target gene - Ct normalizer = ΔCt

ΔCt samples - ΔCt-calibrator = ΔΔCt

Time fold = $2^{-\Delta\Delta Ct}$

Figure 9: The calculations for the values in our line chart are calculated through the $2^{-\Delta\Delta}$Ct-method. [33] Target gene is normalized to reference gene. Resulting number is normalized to the calibrator (0h sample normalized to 0h reference gene).

4.2 Selection of time intervals

The first experiment, conducted by myself in cooperation with Jian Gao, used the cell line (cisplatin resistant E10) and primers (AREG and TBP) from the original experiment. The cells were harvested at 0,5 hour intervals through a 4 hour period, then harvested again at 6 hours and lastly at 8 hours.

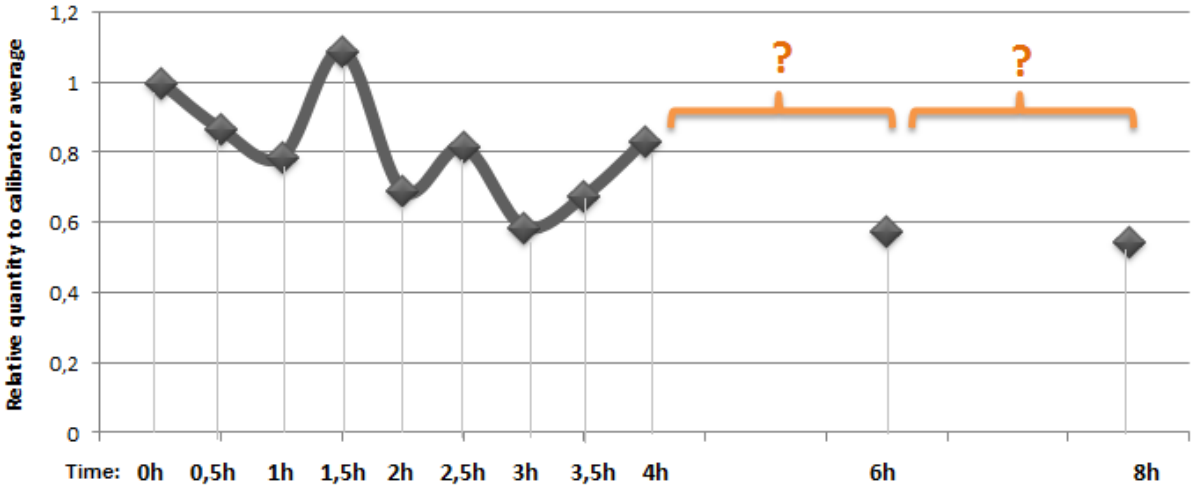


Figure 10: Peaks are observed at 1,5h and 2,5h, but the large intervals between the 4h, 6h and 8h time points exclude any possibility of predicting a pattern in the two areas 4h to 6h and 6h to 8h (marked with **orange '?'**), therefore leaving two empty gaps.

In **figure 10**, two peaks can be visualized at the 1,5h and 2,5h (1,5 hour and 2,5 hour) time points, but due to the 30 minute interval between each interval, it is unlikely that the chart displays the *precise* moments when the graph peaks, as the 1,5h and 2,5h points could be somewhere along the function's increase or decrease. What is even more uncertain is the graph's course past the 4h time point, as the 6h and 8h values alone add minimal or no additional information.

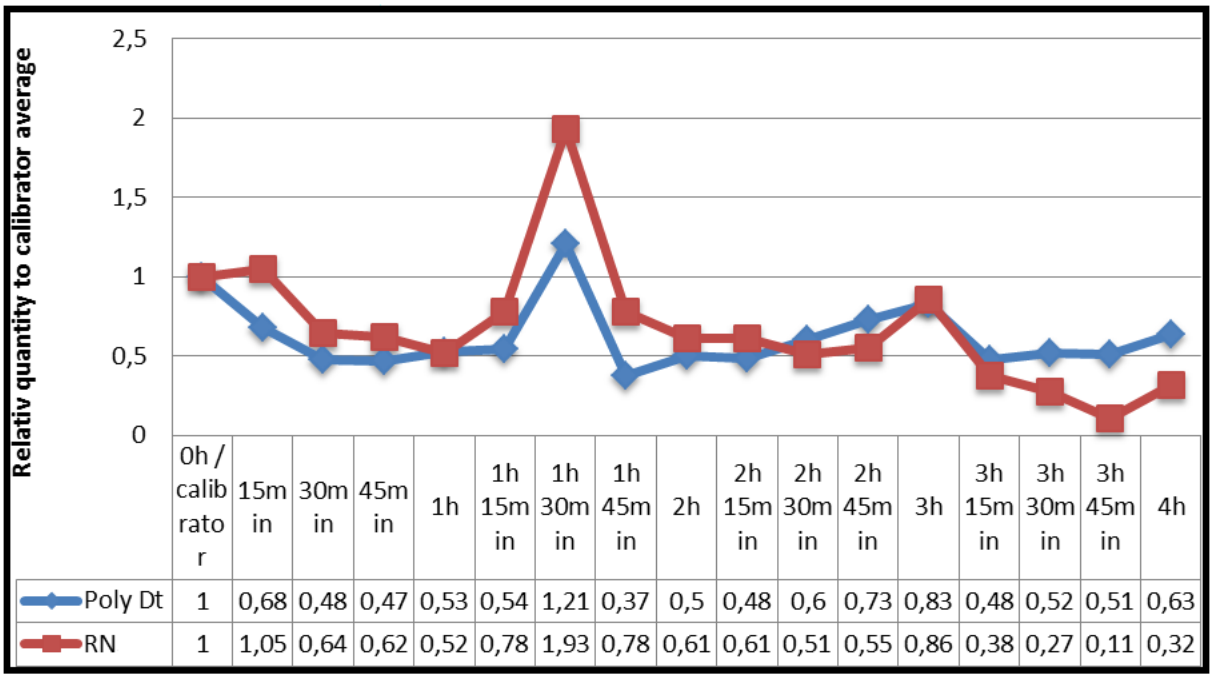
Due to these uncertainties, it was established that the intervals between cell harvesting should be as narrow as possible and happen as tightly as human execution would allow. The fixed intervals for all the following experiments were therefore set at 15 minutes – the shortest span that could practically be achieved.

4.3 Selection of reverse transcription (RT) primers

As previously discussed, the execution of reverse transcription comes with the dilemma of primers. In the reverse transcription kit, both *random nonamers* (RN) and *poly-dT* are

delivered as available choices. Our lab at the Department of Oral Biology, uses RN as the preferred method. However, because the RN primers may transcribe even fragmented denaturized RNA and clutter the results, we ran a parallel RT with both RN and poly-dT.

Two RTs were completed with poly-dT as primer (see **table 3**). The results yielded two graphs with striking pattern heterogeneity. The first fluctuated wildly, while the second showed peak timings resembling that of the RN graphs.



	0h / calibrator	15min	30min	45min	1h	1h 15min	1h 30min	1h 45min	2h	2h 15min	2h 30min	2h 45min	3h	3h 15min	3h 30min	3h 45min	4h
Poly Dt	1	0,68	0,48	0,47	0,53	0,54	1,21	0,37	0,5	0,48	0,6	0,73	0,83	0,48	0,52	0,51	0,63
RN	1	1,05	0,64	0,62	0,52	0,78	1,93	0,78	0,61	0,61	0,51	0,55	0,86	0,38	0,27	0,11	0,32

Figure 11: Parallel reverse transcription of one sample with poly-dT and random nonamers; resulting graphs showed parallelism in fluctuation. Both are AREG normalized to TBP.

The above graph displays a comparison between the second poly-dT AREG/TBP-graph and a typical AREG/TBP-graph reverse transcribed with RN. No considerable divergences could be noted, as the two graphs displayed quite a lot of similarity in fluctuation.

Because the uncertainty regarding the results yielded by poly-dT, it was considered a less stable primer and we resolved to conduct future RTs with RN, which had showed consistency throughout the different experiments.

4.3 Amphiregulin (in E10)

The first four results from the PCRs showed some discrepancy, the charts showed quite contradictive amounts – a few were alike, while others shared no pattern whatsoever. These

can be subtracted from the 18 total PCRs, due to the operator being in training. The later results were more stable and are well represented by steadily showing peaks at time points 1,5h and 3h (**fig.12**). The more consistent results were selected for the data presentation and comparison in later segments.

4.4 EGFR and EGF (in E10)

The possibility of mRNA fluctuation in AREG was well established when it was decided to compare the results with other genes. From a list of primers, two were selected – EGFR and EGF. The first is a transmembrane receptor in the ErbB-receptor family, while the latter is a ligand. The samples were reversed transcribed with RN in the same, parallel process, but in separate PCRs.

	EGFR	EGF	AREG
0h	1	1	1
15min	0,87	0,962	0,491
30min	1,04	0,669	0,809
45min	0,911	0,772	0,523
1h	0,859	1,17	0,681
1h 15min	0,875	0,75	0,526
1h 30min	1,26	4,39	1,77
1h 45min	0,967	1,34	0,771
2h	1,37	0,892	0,768
2h 15min	1,44	1,18	0,678
2h 30min	1,31	0,941	0,72
2h 45min	1,36	1,24	0,78
3h	1,62	1,72	1,11
3h 15min	1,14	2,19	0,877
3h 30min	1,11	1,25	0,536
3h 45min	1,07	0,662	0,52
4h	1,11	0,911	0,566

Table 4: Table containing values, obtained through the $2^{-\Delta\Delta}$Ct-method as described in **figure 9**. Numbers are used as coordinates for **figures 12–16** and will not be repeated in ensuing charts or texts.

4.4.1 Epidermal Growth Factor Receptor (EGFR)

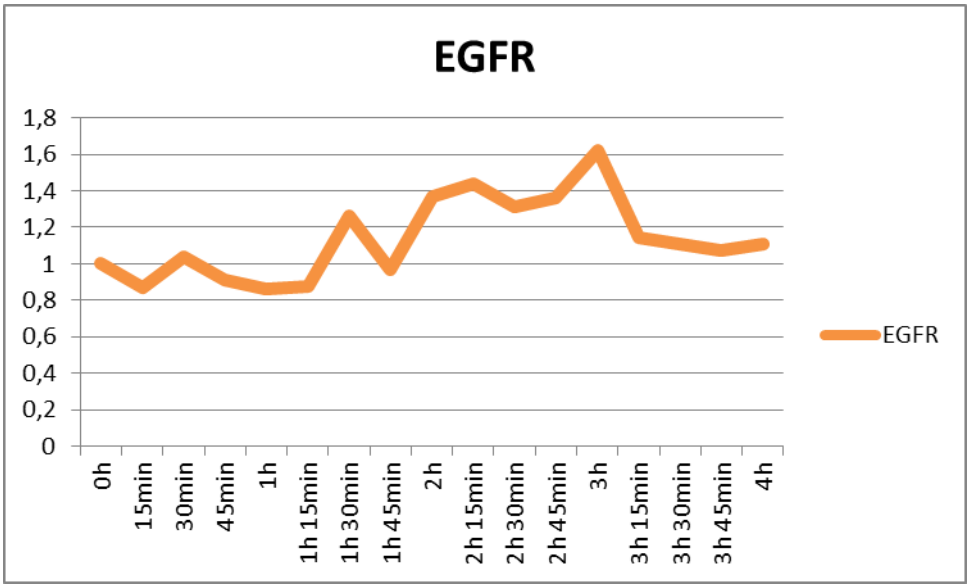


Figure 12: EGFR showed surprisingly little fluctuation and seemed relatively stable. Although one can register a few peaks, the comparatively most prominent peak is at the 3h time point.

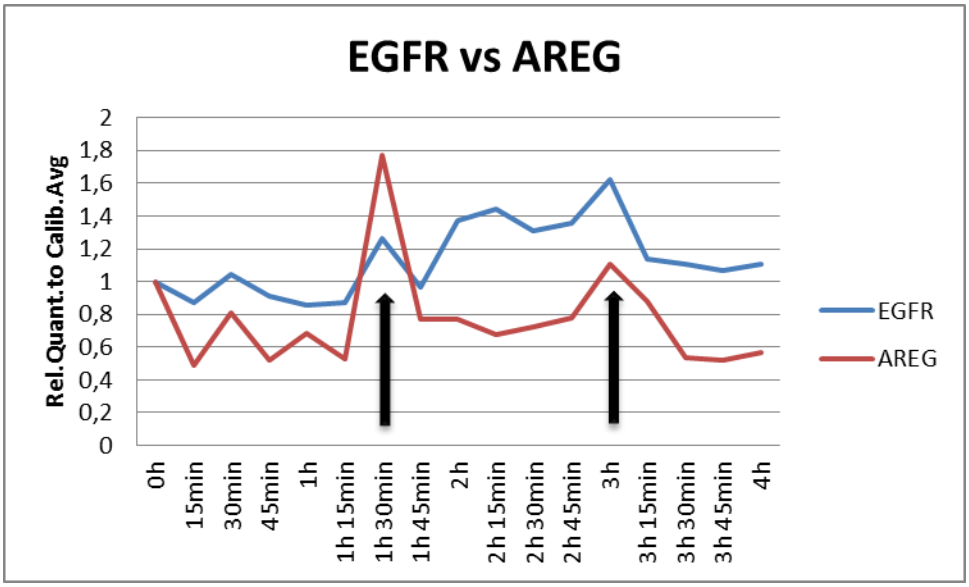


Figure 13: EGFR compared to AREG. Both graphs have coinciding peaks at the 1h 30min and 3h time points. But EGFR shows a relatively more stable increase, making the peaks less prominent, while AREG is better characterized by “spikes”.

EGFR alone shows no prominent "spiky" pattern (**figure 12**). When EGFR is compared to AREG (**figure 13**), the overlapping patterns show peaks at 1,5h and 3h time points, but the AREG peaks are clearly much better defined. When observing this difference, it inspires speculation; could this dissimilarity be caused by the two products' contrasting purposes? As EGFR is a membrane-bound receptor, perhaps a lesser turnover – when compared to AREG which is a ligand – is reflected in its relatively stable mRNA expression over time.

4.4.2 Epidermal Growth Factor (EGF)

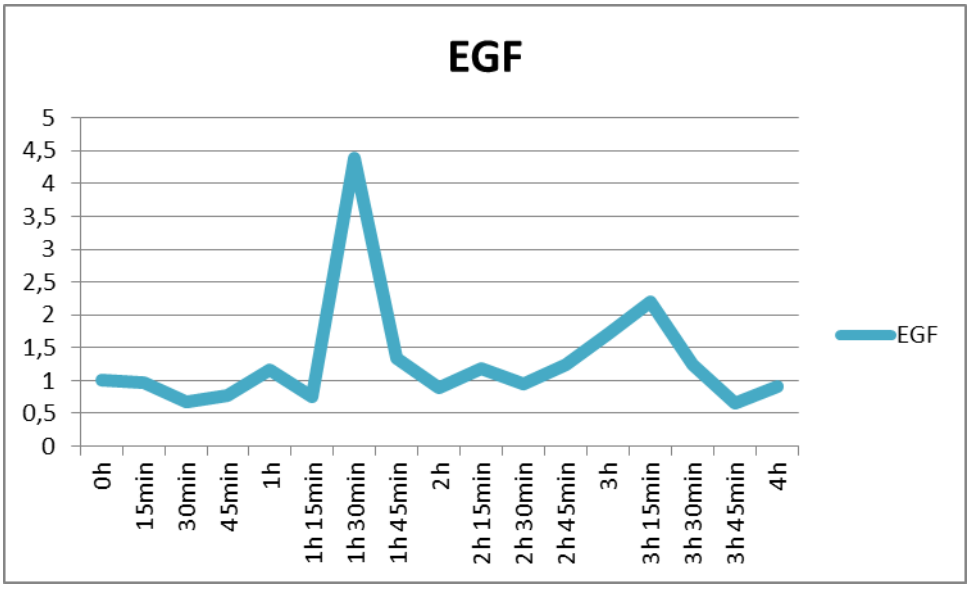


Figure 14: EGF fluctuation, peaks at 1h 30min and 3h 15min.

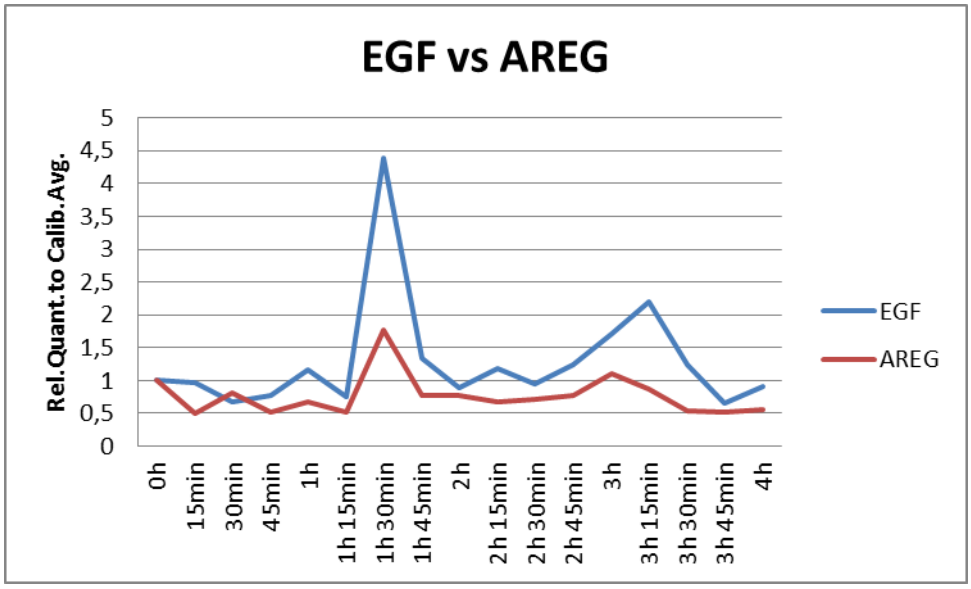


Figure 15: EGF compared to AREG. The first peaks coincide at the 1h 30min. But EGF's second peak is slightly delayed when compared to AREG.

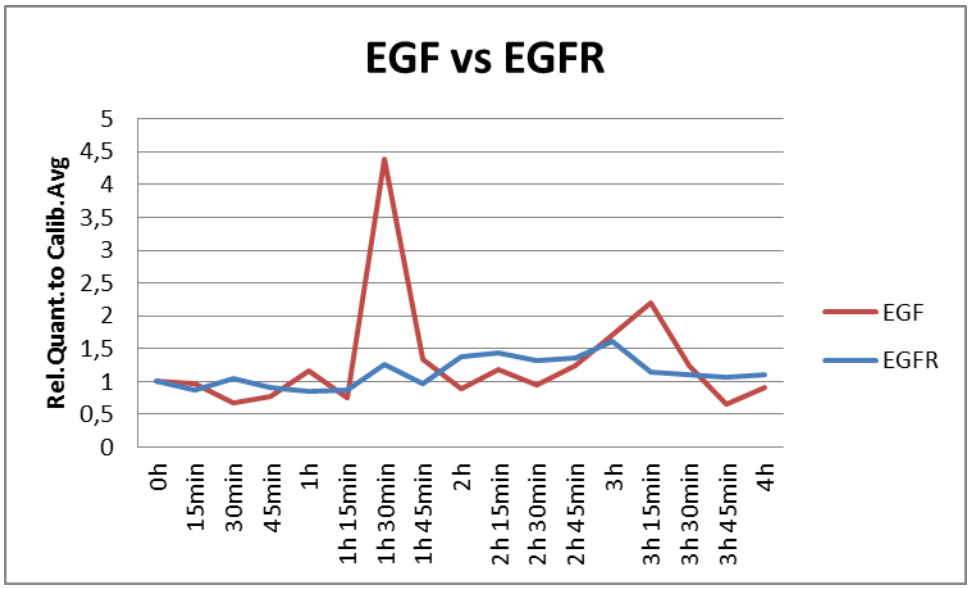


Figure 16: EGF compared to EGFR. The large fluctuations in EGF makes the EGFR seem relatively stable, in comparison.

At first glance the EGF-chart looks a lot more similar to the previous AREG results (**figure 14**). But the first peak is quite large, as it represents an almost 4,5-fold increase in the mRNA compared to the starting 0h calibrator, it trumps AREG's measly 1,8 fold peak (**figure 15**). When EGF is plotted together with EGFR (**figure 16**), their difference in quantity becomes even more evident, supporting the theory that ligands and receptors may have a different expression of mRNA fluctuation – thereby also suggesting that mRNA fluctuation is an expression of some inherent biological system.

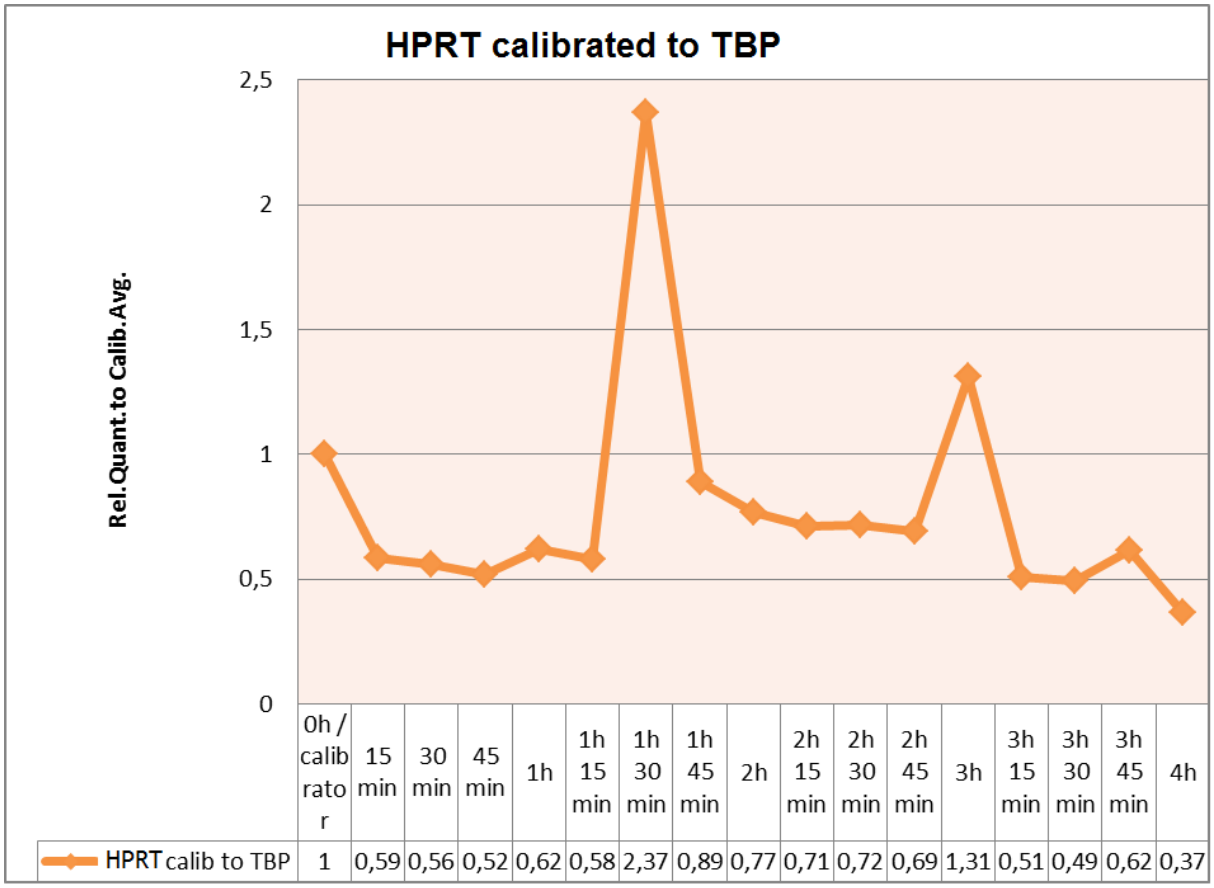
4.5 Control of the housekeeping genes (HKGs)

Upon examining the results, it became almost definitive that mRNA fluctuations did exist. But even if we ascertained its existence, it was still necessary to locate the fluctuations' origin. The three most probable sources are listed below:

- The target gene
- The reference gene (HKG)
- A combination of the target gene and reference gene

To examine this, it was decided to better examine the reference genes. A new HKG was introduced to the experiments and the purpose was to study two HKGs instead of one. As previously established, HKGs are recognized and used for their relative stability in different

tissues and through longer periods of time. The chosen experiment was therefore based on the assumption that a PCR with one HKG as target gene and the other HKG as reference gene should result in a graph displaying less fluctuation than the ones we have previously studied. The hypothesis was based on the belief that HKGs were stably expressed over time, therefore, cyclic fluctuations should be caused by genes other than recognized HKGs. And if this was not the case, perhaps even HKGs increased and decreased periodically.



	0h / calibrator	15 min	30 min	45 min	1h	1h 15 min	1h 30 min	1h 45 min	2h	2h 15 min	2h 30 min	2h 45 min	3h	3h 15 min	3h 30 min	3h 45 min	4h
HPRT calib to TBP	1	0,59	0,56	0,52	0,62	0,58	2,37	0,89	0,77	0,71	0,72	0,69	1,31	0,51	0,49	0,62	0,37

Figure 17: Graph with HRPT as target gene and TBP as reference gene. Results indicate how HKGs also fluctuate in a cyclic manner when studied over time. The peaks occur at time points 1h 30min and 3h – same as the previously shown AREG-fluctuations.

The results from the normalization of HPRT to TBP (**figure 17**) show two peaks occurring at 1h 30min and 3h; this is the same two time points as the peaks in the AREG-function occurred. HKGs show periodic fluctuations and this complicates our data analysis, as one cannot exclude the HKGs as a potential source for cyclic mRNA variations. Whether it is the target genes or HKGs which are causing the changing graphs is impossible to know at this point, but neither can be excluded.

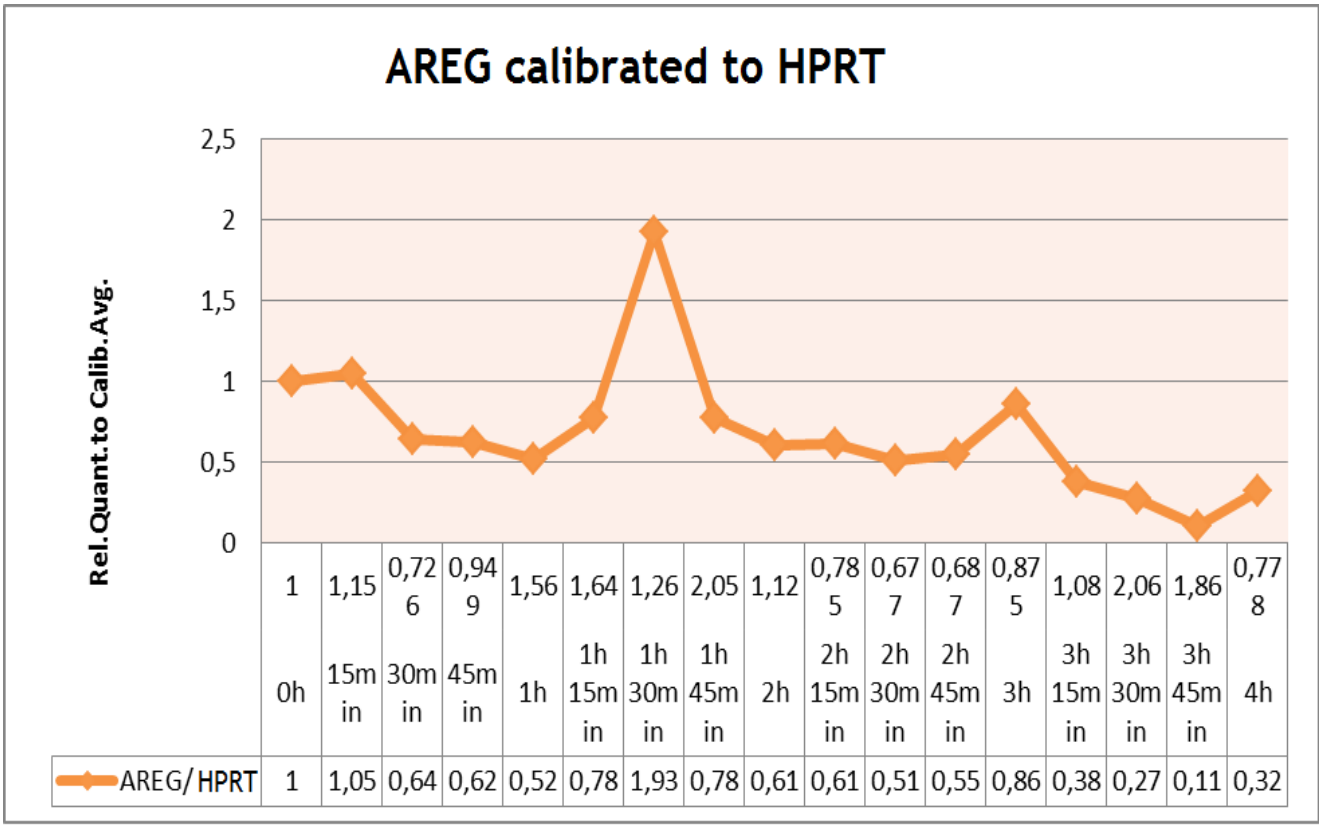


Figure 18: AREG calibrated to HRPT shows no significant difference from AREG was calibrated to TBP. First peak is at 1h 30min and second peak at 3h.

When TBP was excluded and AREG was normalized to HPRT (**figure** 18), the fluctuations' peaks appeared consistently at 1,5h and 3h. It seemed that cyclic variations were unavoidable. Results from the three previous experiments (AREG to TBP, AREG to HPRT and HPRT to TBP) showed the same two peaks occurring at the same two time points, it makes us wonder: Which gene is actually causing the fluctuations, is it one, two or all three? Or is it none, but simply an experimental error?

To better understand HKG-variation, the Ct-values for the two HKGs through a four-hour period are displayed in **figure 19**. Each graph represents one PCR, so every function is singular and separate from the rest. **Figure 19** is different from previous results as resulting numbers have not been normalized nor calibrated. The unprocessed HKG-figures (**table 5**) are not strikingly aberrant and it is unlikely that fluctuations from the previous plots could be initiated by the HKGs alone.

	HPRT from TBP-HPRT 19.8	HPRT from AREG-HPRT 19.7	HPRT from AREG-HPRT 22.7	TBP from TBP-HPRT 19.8	TBP from TBP-HPRT 19.8	TBP from AREG-TBP 29.7	TBP from EGF-TBP 27.7	TBP from EGFR-TBP 24.7
0h	24,81	21,16	21,95	27,82	27,82	29,68	28,29	27,81
15min	26,79	20,73	20,64	29,02	29,02	29,65	28,74	28,14
30min	26,32	20,96	21,47	28,48	28,48	29,88	29,18	28,32
45min	26,84	20,27	21,61	28,87	28,87	29,84	28,85	28,37
1h	26,61	20,3	21,88	28,94	28,94	29,96	29,04	28,22
1h 15min	26,26	19,58	21,65	28,49	28,49	28,81	27,99	27,83
1h 30min	25,45	20,52	21,4	29,7	29,7	29,8	29,91	28,74
1h 45min	26,74	19,77	21,23	29,58	29,58	29,6	29,58	28,5
2h	27,01	18,92	21,45	29,66	29,66	29,86	29,24	28,82
2h 15min	27,06	20,07	21,21	29,59	29,59	30,14	29,01	28,88
2h 30min	26,81	19,07	21,27	29,35	29,35	30,1	29,14	28,97
2h 45min	27,56	20,05	20,99	30,05	30,05	30,41	29,48	29,39
3h	27,05	19,83	21,83	30,46	30,46	29,45	29,28	29,16
3h 15min	27,6	19,48	21,4	29,67	29,67	30,48	29,91	29,35
3h 30min	28,27	19,72	21,15	30,27	30,27	30,18	29,37	29,26
3h 45min	28,66	18,94	20,89	30,95	30,95	30,71	29,5	29,17
4h	28,42	19,19	21,25	29,86	29,86	29,72	29,64	28,96

Table 5: Table containing Ct-values of the samples presented in **figure 19**.

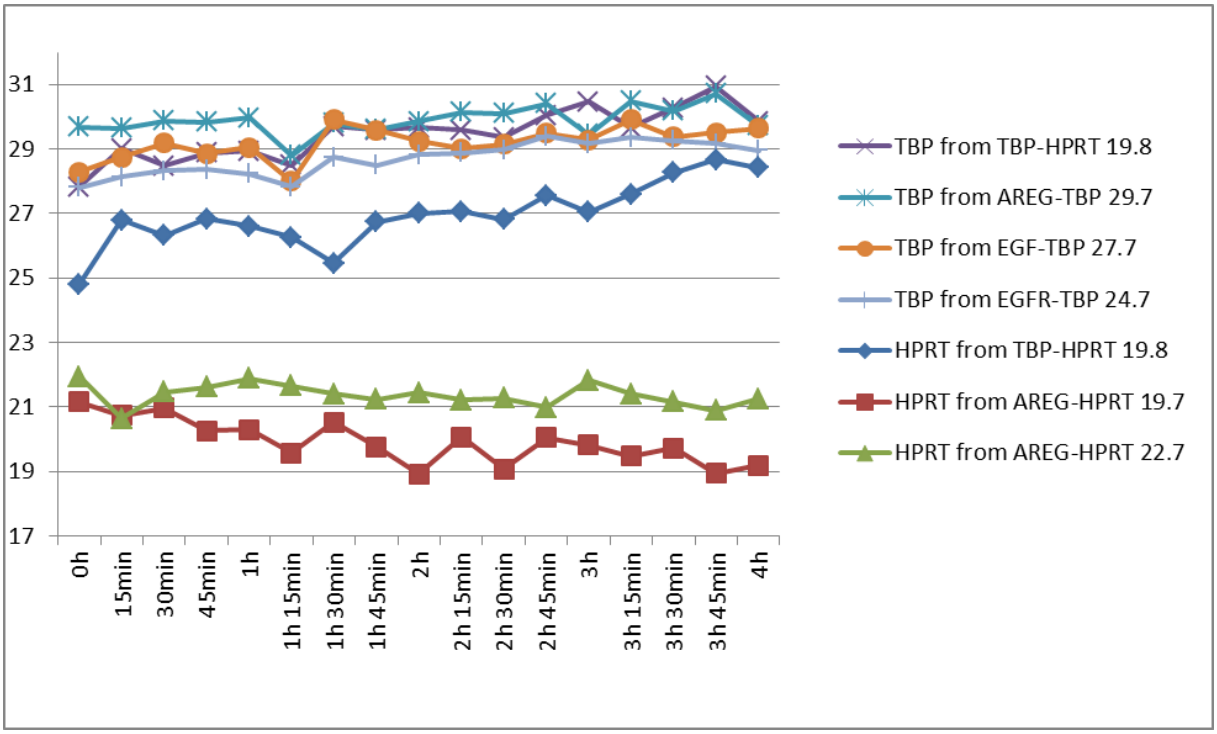


Figure 19: Ct-values of HRPT and TBP, respectively, from different PCRs. The numbers represents the dates when the PCRs were completed (year 2013).

	AREG	EGF
0h	1	1
15min	1,15	1,11
30min	0,726	0,785
45min	0,949	1,18
1h	1,56	2,86
1h 15min	1,64	1,14
1h 30min	1,26	1,16
1h 45min	2,05	0,455
2h	1,12	0,114
2h 15min	0,785	0,164
2h 30min	0,677	0,215
2h 45min	0,687	0,291
3h	0,875	0,166
3h 15min	1,08	0,118
3h 30min	2,06	0,97
3h 45min	1,86	4,41
4h	0,778	0,144
4h 15min	0,476	0,131
4h 30min	0,607	0,183
4h 45min	0,614	0,355
5h	1,21	0,191

Table 6: Table containing values, obtained through the $2^{-\Delta\Delta}$Ct-method as described in **figure 9**. Numbers are used as coordinates for **figures 20-22** and will not be repeated in later segments.

4.6 Comparing to cell line C12

Up to this point, the results have indicated the presence of fluctuations in different genes, but all cells were cultured from *one* cell line – E10. To examine whether mRNA fluctuations were a general biological phenomenon, the PCRs were repeated in the cisplatin sensitive cell line C12. The experiments were completed towards the project's end, which is the reason for not including more repetitions to ensure greater accuracy.

To extend the study, the total time length for cell extraction was prolonged by one hour, from 4 hours to 5 hours. AREG and EGF were selected as target genes and both were normalized to TBP. For listed details and accurate numbers, see **table 6**.

The acquired results for cell line C12 are shown in **figure 20**. In **figure 21-22**, AREG and EGF from C12 are analyzed with matching experiments from cell line E10. Results representing E10 have been shown in previous sections and to ensure the most accurate comparison, parallels share both target gene primers and reference genes primers.

By comparing the graphs, it is quite simple to notice dissimilarity in the graphs' development. A possible explanation for this may be technical inaccuracies, but the graphs' irregularity is still noteworthy. Nevertheless, the inherent fluctuations can be recognized in both genes.

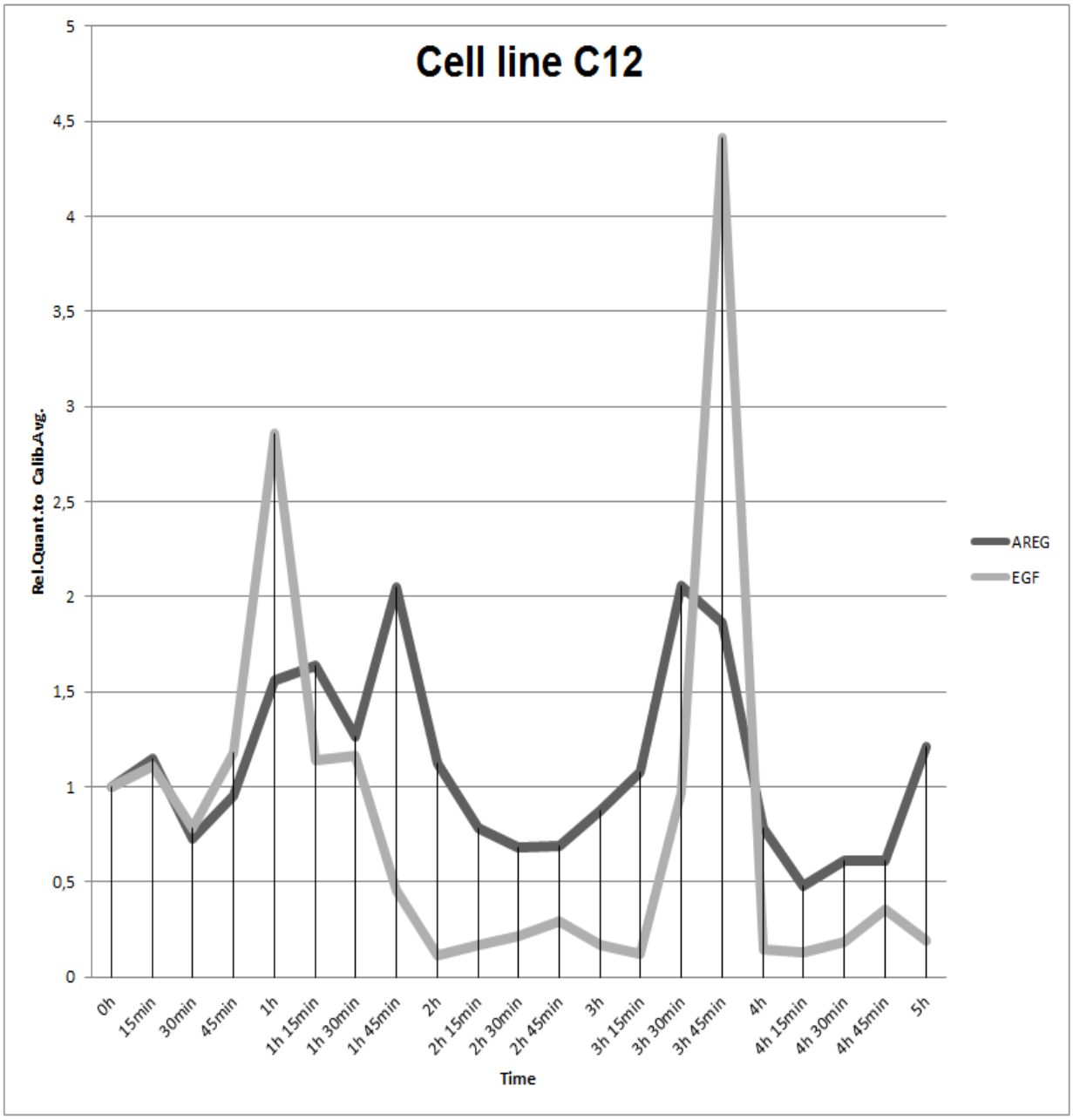


Figure 20: Graph illustrating normalized Ct-values for AREG and EGF, acquired from cell line C12. Both are normalized to TBP. The exact values are listed in **table 6**. The highest peaks for AREG are found at 1h 45min and 3h 30min. The highest peaks for EGF occur at 1h and 3h 45min. The graph patterns develop in a similar manner with peaks and dips. There is a noticeable dissimilarity of pattern progression as the peaks occur at greatly varying heights and at different time points.

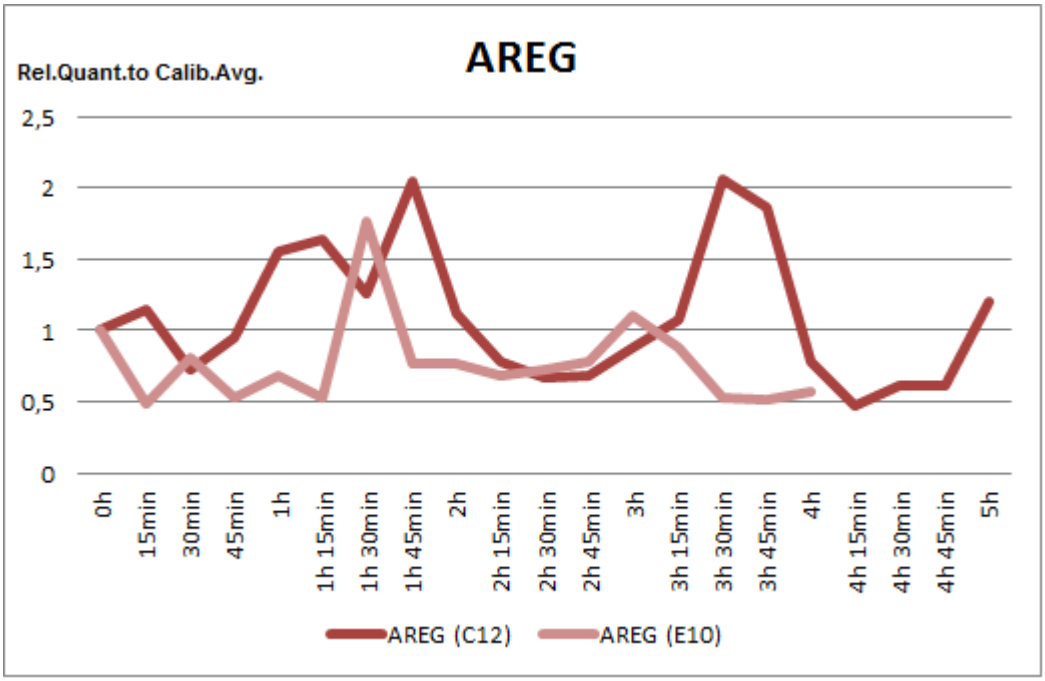


Figure 21: Comparison of AREG from the two different cell line (E10 and C12) shows great disparity of the patterns. Details for AREG from E10 can be found in **table 4**. The graph is also depicted in **figure 13**.

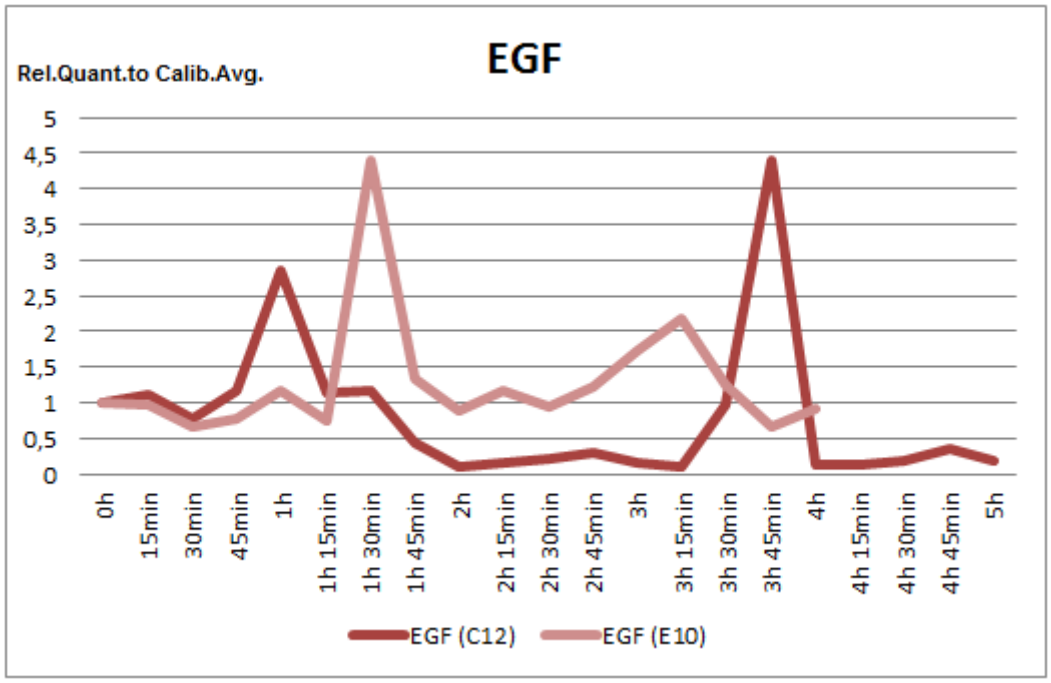


Figure 22: Comparison of EGF from the two different cell line (E10 and C12) shows great disparity of the patterns. Details for EGF can be found in **table 4**. The graph is depicted in **figure 14-15**.

4.7 Summarizing the results

The results above show how the fluctuating patterns differ between the different genes and also between the two cell lines. In all the presented graphs, there is an unmistakable individuality in each graph and its pattern course. The fluctuations indicate the changes in each gene's mRNA concentration and, by extension, that gene's expression. By examining these results, it seems that mRNA expression varies in a cyclic manner. If this is the case, the study and understanding of mRNA regulation becomes extremely complicated. However, this indication is only a hypothesis and the hypothesis is only tentative. We cannot exclude the possibility that the mRNA fluctuations are stochastic events. Our results are only suggestive and are by no means definitive. Before any conclusion can be reached, the complex nature of mRNA needs to be better understood and further research is still needed.

Part 5: **Discussion**

5.1 Oscillations in literature

Many researchers have published and discussed the fluctuations of mRNA but the topic has received surprisingly little attention. While most remain unaware of this observation, studies which discuss this matter have already coined it with the term "mRNA oscillations". Many methods for protein quantification and mRNA analysis exist and different techniques are used by various researchers. While many agree with the existence of mRNA oscillations, some also suggest that similar oscillations take place in protein expression – thereby further confirming its relevance for gene expression.

Whereas we used RT real time quantitative PCR to study mRNA fluctuations, others have used different methods and observed similar developments. [55] In a study by Geva-Zatorsky et al. [56], individual cells were studied by time-lapse fluorescence microscopy movies. They captured images of the cells' fluorescence every 10-20 minutes and discovered fluctuating protein levels in different cells – some changes resembled oscillations, while others did not. The authors observed that DNA damage was followed by oscillatory behavior in the p53 and Mdm2 protein systems, and that the fraction of oscillating cells increased with an increasing dosage of gamma irradiation – an observation confirmed by others. [56] Lev-Bar Or et al. discovered an oscillatory response in various cell types to ionizing radiation and suggested that oscillations were a response to a stress signal and allowed for damaged DNA to be repaired. [57] On the other hand, in a single-cell experiment by Hat et al., the p53 (and NF-κB) system displayed oscillations which were insensitive to the strength of stimulation. [58]

The above examples illustrate how inconclusive the studies regarding oscillatory gene expression are, although several sources agree that such fluctuating behavior exists for both mRNA and protein. Both *stochastic* and *cyclic* behaviors may produce oscillating mRNA levels and protein expressions, yet there is still no *conclusive* explanation for the mechanisms regarding oscillations; their initiation, regulation or their effects on cellular protein concentration and gene expression. [55, 59]

5.2 Gene fluctuations – stochastic or cyclic?

Variation in mRNA concentration has been described as both stochastic fluctuations and temporal oscillations. [55, 59] The stochastic variations have been described by Rodríguez et

al. They discovered random fluctuations in the mRNA, which were amplified during translation and generated sustained pulses of protein expression. [59] Whereas Tiana et al. described temporal or cyclic oscillations, where oscillations reoccurred after a certain period of time. The temporal oscillations were further subdivided as *ultradian* oscillations – which were observed with a few hours' time interval – and *circadian* oscillations which echoed with 24 hour rhythms. [55]

Most authors regard these mRNA-protein fluctuations to be part of a negative or positive feedback loop system. [55, 59]

5.3 Possible mechanisms

Feedback mechanisms

Negative and positive feedback mechanisms are common in cellular systems. It exists by having one variable which either activates or represses its preceding variable – this creates a positive or negative feedback mechanism, respectively. [55] The reason why the negative feedback loop is such a widely renowned explanation for oscillations, is because oscillatory behavior is described in many aspects of biology and biological systems are frequently regulated by this type of negative feedback mechanism. Examples of such regulation can be found in nerve signals and circadian clocks. [60]

Others, on the other hand, believe the oscillations are due to a positive feedback mechanism. Lev Bar-Or et al. have observed fluctuations in relation to stress signals in the p53-system. These authors suggested that the pulses in protein expression are initiated and sustained by damage; that the spikes are continuously sent until damage is repaired. If the damage is extreme, the amplitude and duration of the pulse will induce p53-overexpression, and thereby apoptosis. [57] Geva-Zatorsky et al. also observed that the amount of oscillating cells increased along with an increase in gamma irradiation dose, yet this could not fully explain the variability:

> **"Deterministic simulations cannot capture the variability in the oscillation amplitudes observed in the cells. We therefore added internal stochasticity to the equations."** (Geva-Zatorsky et al.) [56]

Noise and stochasticity

Some cells studied by Geva-Zatorsky et al. did not express oscillatory fluctuations; instead they displayed slow, varying fluctuations. [56] Rodríguez Martínez et al. have proposed several models for such fluctuations, but find them insufficient. Instead, they support the idea of a *noise element* during protein production which creates the variability in oscillations, meaning that they accept the presence of stochasticity in their theory. They have developed a fully stochastic model in mRNA expression, described and tested through mathematical models, and propose that the fluctuations may be inhibited by regulatory RNA (regRNA), such as microRNA. [59]

Despite the ongoing debate regarding the mechanism of these oscillations, many authors [61] agree that:

> "**[…]oscillations in protein concentrations or gene expression levels are related to the presence of at least one negative feedback loop in the regulatory network."** (Pigolotti et al.) [62]

Pigolotti et al. pronounce oscillations as a physiological process; an idea which disagrees with the abovementioned "response to DNA damage". [62] This discrimination affirms *our* observation, because the oscillations in our results occurred without provocation by any external stimuli. Our interpretation of the foregoing data strongly concurs with the hypothesis that a feedback mechanism is the key source for fluctuations in the mRNA.

Any mRNA regulatory mechanism could be capable of gene expression modification but, as suggested previously, a likely suspect is the previously mentioned regRNA and particularly the microRNA regulatory system.

5.4 microRNA

microRNA (miRNA) has a great impact on the transcription of mRNA and a very likely regulatory mechanism – a possibility authors researching the field of mRNA fluctuations are becoming increasingly aware of. Due to the miRNA's diverse ability to participate in feedback and feed-forward mechanisms, it is viewed as the next target in the study of gene oscillatory behavior. [59]

miRNAs are short non-coding RNAs, ranging from 20 to 24 nucleotides in length. They have important gene regulatory functions and can modulate both mRNA stability and translation.

[63] By Treiber et al., the mRNAs are referred to as “key regulators of literally all cellular pathways”. [64] The miRNAs participate in gene regulation and can induce either down-regulation or up-regulation of translation. [65]

5.4.1 Down-regulation

The repressing acts of miRNA are primarily through repression of mRNA translation or degradation. The process is complex and the exact mechanics regarding how miRNA chooses their target genes are still unknown. [65] Several pathways for repression has been described, but in general terms, the inhibition of protein synthesis through miRNA is either by repressing translation or by deadenylation and destabilization of mRNA target. [66, 67] They have many different functions:

- *Repression of translation initiation.* 3’ UTRs can be targeted by miRNA, causing repressed initiation of translation, by preventing ribosomal loading of mRNA. [66, 68]
- *Post-initiation repression.* miRNA initiated repression can occur during translation, either as the polypeptide chain is made or after its completion. miRNA initiates decay of newly synthesized polypeptide chain. Whether the process is post- or co-translational is under discussion. [65, 67]
- *mRNA decay.* miRNA associated with proteins in the AGO/eIF2C family can form miRNA-induced silencing complex (miRISC). miRNA guides these complexes to mRNA. A partially complementary base pairing leads to sequence-specific repression or mRNA degradation. [63-65]
- *5’ cap.* Some translation is cap-dependent; to initiate translation, the recruitment of initiation factors at the 5’ cap is necessary. miRNA may interfere with the recruitment or function of these factors. [66]
- *Storage or degradation.* miRNA association with P-bodies and Stress Granules (SGs) seem to induce silencing of mRNA by preventing targets from reaching the ribosomes for translation. The silencing is either through degradation or storage. [65]

5.4.2 Up-regulation

Activation or up-regulation of mRNA translation through miRNA is a new discovery, but it is only reported under special conditions, like during the cell cycles G_0/G_1. Repression has also

been observed in G_1-phase, so a more generalized premise for up-regulation is not yet discovered. [66, 68]

5.4.3 miRNA – reflected in mRNA?

The very first miRNA was identified approximately 20 years ago. Today, about 900 miRNAs have been identified. [69] miRNA is recognized for its wide range of regulatory functions, and its importance in cellular mechanisms is becoming increasingly clear. miRNAs are identified as both tumor suppressors and oncogenes; their role and participation in oncogenesis is therefore a subject of widespread interest. [70]

To summarize, although the connection between mRNA fluctuations and miRNA is not yet evident, the mere possibility of a connection inspires important questions. The *first* question which needs to be answered is whether miRNA is the regulatory mechanism that creates the mRNA peaks. The *second* question, which perhaps is even more important, is whether fluctuations in mRNA can be mirrored by fluctuations in miRNA. Both points are essential to understand how mRNA oscillations are regulated. Research in this field is undoubtedly needed.

5.5 Implications of the results

The oscillations in the mRNA resulted in greatly varying patterns. Dips and peaks were noted for every single result, but the time point for an occurring peak and its peak height showed remarkable inconsistency. The most noteworthy result showed an amplitude which expressed an mRNA-level *seven times* greater than the 0h calibrator, expressing how the extent of mRNA fluctuation truly can be extreme.

To illustrate the implications of the collected data, **figure 23** shows EGF's pattern through a 4h time period. Horizontal line marks the threshold as the samples reach a concentration that shows a 50 percent increase in comparison to baseline, in other words, the concentrations are *at least* 1,5 times the 0h value (1,5 x 0h). These areas are marked with yellow and apply for intervals 1h 15min to 1h 45min and 3h to 3h 30min, this amount to a total time period of ~30 minutes for the first yellow area and 30 minutes for the second area. The total amount of time in which the mRNA increases from 1,5 to 4,5 times the original baseline concentration becomes approximately 1 hour – ¼ of the total 4 hour time period.

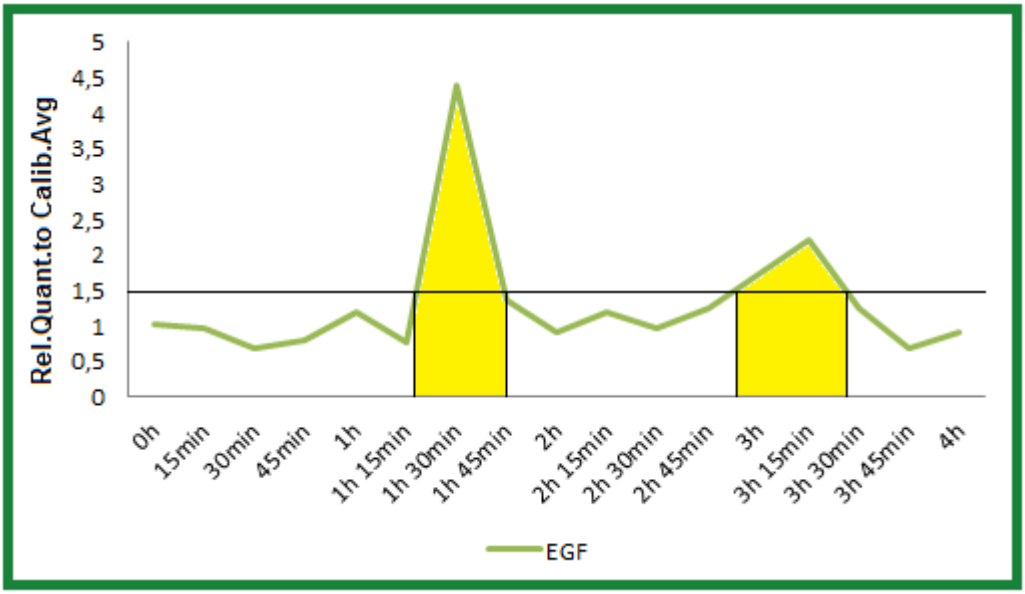


Figure 23: The fluctuation of EGF over a 4 hour period results in two prominent peaks. The threshold value, the horizontal black line, is set at 1,5 x the 0h baseline concentration, meaning that graph areas which cross this threshold, display an mRNA-concentration which is equal to or higher) than the original value of mRNA. Graph areas which is ≥ 1,5 x 0h baseline, in comparison to 0h baseline, is marked in **yellow**. Times which correspond to these concentrations are the ranges 1h 18min to 1h 45min and 3h to 3h 30min.

Under most conditions, mRNA quantification with RT-qPCR is executed at a single time point. By using the data above to illustrate, one sample could show a concentration from any time point between 0h and 4h, in the above graph. It could have a concentration similar to the 30min time point (0,75 x 0h value) or the 1h 30min time point (4,5 x 0h value). Regardless of the resulting number, it would be assumed to be the "truth". But except for the two peaks (**yellow** area in **figure 23**), the rest of the graph stays relatively stable, ranging from 0,5 to 1,5.

In conclusion, ¾ of the samples represent stability, while ¼ show aberrant fluctuations. When a *specific* mRNA sample is quantified and the value from that *specific* time point is used, that number is regarded as a *mean* level of mRNA concentration. The mRNA levels are assumed to be stable over time, but this data clearly contradicts this. It proves how mRNA levels for a gene cannot be based on one single measurement. Two different genes cannot be compared by two separate mRNA quantifications – one measurement may be a "peak" value and the other may be a "bottom" value. The total mRNA production for a target gene is expressed by the ***area under the graph*** because the levels are not stable, they fluctuate over time.

5.6 Correlation between mRNA and protein

The variation in mRNA levels may explain the discrepancy in translating mRNA values to protein levels. Deviations in the levels of mRNA when compared to protein have been registered among many authors. [71, 72] But these results have largely been attributed to mRNA variations, post-transcriptional- and post-translational regulation, as well as errors in measurement. [72]

Some results between mRNA and protein correlate very little. [73] Oscillations may be the reason for this. This sheds doubt on any modern technique using mRNA to study protein expression; results verified by such techniques are jeopardized. This includes examination of different expressed genes with microarray based mRNA expression systems that detects the expression of more than 40 000 different genes at a single time point. If all genes had similar fluctuations, approximately 25 percent of the genes will be within one of their peaks and show a peak-related expression level which may not reflect their effect on protein expression at all. Thus, conclusions based on mRNA expression studies must be supplied with protein expression assays, before the results can be trusted.

Part 6: Conclusions

The presented hypothesis is that mRNA levels show periodical oscillations. We do not believe these to be stochastic, but a part of a cellular feedback system, perhaps regulated by miRNA. Several authors support the theory about fluctuations in mRNA concentrations and gene expression, though they do not entirely agree regarding the underlying causes and whether the occurrence is coincidental or regulatory.

Although we have not yet found common ground regarding the mechanisms, we should all be able to accept its presence. The fluctuations in mRNA levels greatly complicate the research targeting gene expression. It also questions the validity of qPCR – a tool widely used for capturing snapshot events and uses this to draw conclusions about gene expression, protein synthesis and RNA levels. But if the mRNA fluctuates, the snapshots may depict an aberrant moment – a peak or it may depict a bottom – instead of a desired average. In either case, comparison between different cell lines, or even within one cell line, cannot hold any validity.

If these results can be generalized and applied to most other mRNA expression behaviors, it is a paradigm shift, as mRNA quantification at a single time point may not reflect the amount of mRNA the cells translate nor the cells' level of protein synthesis. Only further research may shed light upon this field. With this presented work, I would like to encourage this subject, mRNA oscillatory behavior, to be better and thoroughly studied, in the near future.

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