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Evaluation of mRNA Markers for Estimating Blood Deposition Time

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Abstract

RNA analysis provide insight into diseases, molecular identification of body fluids and mechanisms leading to death and might develop into a valuable tool for identification of the cause of death in forensic pathology. Further potential uses are the determination of the age of wounds and the post-mortem interval. In this proof-of-concept pilot study clarifies and explains principles, applications and methods by offering a comprehensive and complete overview of using mRNA markers for estimating blood deposition time which can help to evaluate the time of the crime in forensic RNA work. The study presented in this thesis aimed to estimate the time passed since blood stains found in the crime scene by calculating the time of deposited blood using particular mRNA markers and unravelling one of the principles of at what time – when during the day or night a biological evidence was left at the scene – by applying the insights from circadian biology to some open forensic cases. By analyzing 4 candidate mRNA markers expression in peripheral blood samples collected from 29 health males. Blood samples were collected from healthy persons for the duration of the 24 hours' day/night interval under four different groups, i.e. night/early, morning early morning/morning, morning/afternoon and afternoon/night. This study identified 2 mRNAs with statistically significant expression rhythms which are MKNK2 and PER 3. It's found that, in general mRNA-based estimation of time categories was less accurate.

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The value of mRNA was demonstrated for blood deposition timing and introduced a statistical model for estimating day/night time categories based on molecular biomarkers, which shall be further validated with additional samples in the future.

Keywords: Circadian clock; circadian rhythms; clock genes; oxidative stress; sleep deprivation.

1. Introduction

With existing technologies and methodology in forensic science, a major quantity of genetic information might be acquired from an individual biological trace found at a scene. Obtaining a DNA profile is not the only possible information that can be acquired from stain's donor, but novel methods and techniques are also being progressed to evaluate body fluid source depending on RNA techniques. In addition to determine physical characteristics such as eye color, hair color, height, skin pigmentation, relative age and weight [1]. The probative knowledge acquired from the trace samples might assist forensic scientists in cases in which there is no known suspect. The ability to determine when a biological stain was deposited would aid in the reconstruction of a timeline of events associated to a crime that may be limited to eyewitnesses. During a crime, evidence is deposited through interactions between the perpetrator and the victim. It is often difficult to determine if the DNA evidence found at the scene was left during the crime or deposited during a previous interaction. Through short tandem repeat (STR) analysis, the person from whom the sample originated can be determined; however, to link or exclude the source from the crime requires a witness or other evidence. When proof of the time of deposition is lacking, the reliability of the evidence itself may be called into question in a court case. If the suspect and victim suspect have a history of previous personal contacts, it is not uncommon for the suspect's DNA to end up on or around the victim or vice versa. It may be argued that the DNA sample was left prior to the commission of the crime due to social relationships between suspect and victim. Being able to distinguish when a biological sample was deposited would help to resolve this dilemma.

1.1 Techniques developed to determine the age of a bloodstain

Circadian rhythms are entraining able, endogenously generated alteration with an interval of around twenty-four hours available in nearly all organisms, counting humans. Recently, it has been shown that each cell has its molecular clock consist of a network of oscillating mRNAs and their protein products [1-2]. Many biological procedures show major daily alteration including behavior and activity, heart rate, wake-sleep cycle between others and hormone secretions [3-4]. Awareness about the fundamental molecular mechanism of these processes and their components could afford an excess of possible rhythmic biomarkers also for forensic cases. In theory, such rhythmic biomarkers might be valuable for molecular age determination in two different forensically ways: determining the interval of death and determining the deposition time of biological evidence which found in a crime sight. The latter might provide a molecular explanation analysis permitting DNA identification of donors' sample to be associated with criminal that is extremely appreciated meant for forensic investigation [5]. The initial approach to evaluate evidence deposition age via incorporating a chronobiological principle into forensic science field was published since 2010 [6]. In that proof-of-principle experiment, the twenty-four hours' profiles of two

circadian hormones, cortisol and melatonin, were reproduced in saliva and little blood samples collected during the day. The possibilities of applying this method in forensic cases were also shown it requires in little quantity of samples and utilizes commercial assays together with standard laboratory tools. The time frame predictable with those two hormones is, though, limited to either early morning hours in case of cortisol or late night in case of melatonin [6], and both of them are known to be influenced by surrounding factors [6]. Thus, searching for extra biomarkers with rhythmic changes in quantity which eventually might confirm important voluble for increasing the accuracy of biological evidence to pinpoint the deposition time in forensic applications, is optimistic. Evaluating the time of criminal using biochemical markers, such as melatonin, has been discussed earlier [7-8], and newly a proof-of-principle research proposed using of microRNAs (miRNAs) for that reason [9]. MicroRNAs are of enormous significance for different forensic investigation applications [5], because of their small extent, higher resistance to degradation with comparing of mRNA, tissue specific expression, and were earlier proposed as appropriate markers for forensic body fluid recognition [10-11-12]. Recently various microRNAs have implied in the directive of circadian rhythms [13-14-15], and several of them were exposed to display diurnal expression changes in various tissues, such as suprachiasmatic nucleus or liver [14-15].

1.2 Circadian Biology

A circadian rhythm can be defined as any biological procedure that can show an endogenous, entrain able alternation of about twenty-four hours. These twenty-four hours' rhythms are determined by a circadian timer, and they have been broadly perceived in fungi, plants, cyanobacteria and mammals [16]. The expression circadian originally from the Latin circa, which mean approximately or around and diēm, define as day. The official research of biological time rhythms, for example daily, tidal, annual rhythms, weekly and seasonal, is called chronobiology. Procedures with twenty-four hours 'alternations are more usually called (diurnal rhythms). They must not use circadian rhythms except their endogenous nature is confirmed [17]. This was the first evidence for the existence of an internal biological clock. In the following years, circadian rhythms were found in most of existing life forms, from single cell to multicellular organisms, proving that they are conserved throughout evolution.

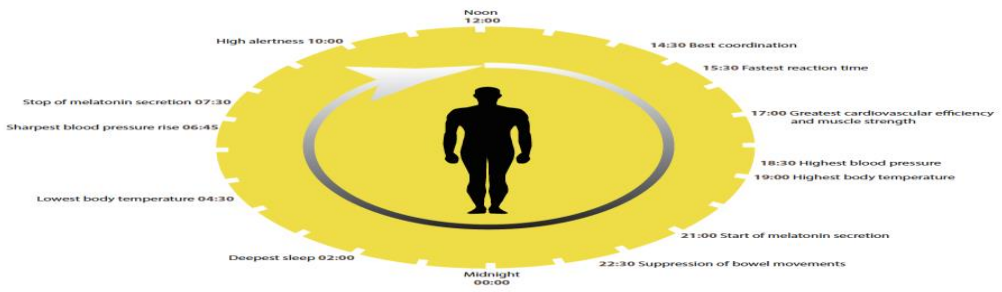

Figure 1: Circadian rhythms are reflected in various physiological as well as behavioral processes in humans (School of Biological Sciences, Royal Holloway University of London).

Circadian rhythms can be illustrated by three inherent properties. First of all, they are self-sustaining or free-running, persisting in the lack of any external time signs (i.e. light/dark cycle) with a period of approximately 24 h. Secondly, they are susceptible to resetting (entrainment), by external time cues (zeitgebers) such as the daily

light/dark cycle, temperature or food. Lastly, the circadian rhythms are temperature compensated, which means that their period length is constant over a wide range of physiologically relevant temperatures. All those features contribute to a robust, yet susceptible to fine-tuning, circadian system. In mammals, the circadian systems have a hierarchical organization. They are comprised of the "master clock" which found in the suprachiasmatic nuclei (SCN) in the hypothalamus, that synchronizes the phase of other clocks called the peripheral clocks, present in practically all tissues outside the SCN. However, all circadian systems begin with the molecular circadian oscillators present in almost all individual cells, consisting of a set of genes and their protein products that generate the circadian rhythms through changes in their expression. Even though circadian rhythms are endogenous, they are entrained to the local surroundings through external cues known as zeitgebers or in another term time giver, which include of light, redox cycles and temperature [18].

2. Aim of The Work

The core aim of forensic investigation is to discover when, how the crime happened and who did the crime. Who was the offender that committed the criminal offense; in what way or how acted the crime scenario; and at what time or when acted the offence occur. Now days, there are techniques which facilitate the investigators and researchers to predict the two questions, the how and the who. The when is still in demand of developing precise and reliable tests. Study presented in this thesis aimed to:

1. To determine at what time – when during the day or night a biological evidence was left at the scene – by applying the insights of circadian biology simulating forensic cases.
2. To validate the role of mRNA markers to detect the time passed since blood stains were deposited in the crime scene (blood deposited time).

3. Matrials & Methods

3.1 Methods

In general Blood samples were collected from twenty-nine healthy individuals during the 24h day/night period under four different groups, i.e. night/early morning, early morning/morning, morning/afternoon and afternoon/night controlled sleep-laboratory conditions. Then isolated the mRNA. Moving on performed reverse transcription reaction by used polymerase chain reaction to convert mRNA to cDNA. After that, quantified cDNA using four different primers. Finally, RT-PCR techniques applied to the samples to end up with analyzed the data and performed statistical prediction modeling.

3.1.1 Sample Collection

Twenty-nine blood samples were collected from healthy individuals living in Abu Dhabi-UAE in EDTA tubes and stored at -80℃ until use.

3.1.2 Rna Isolation And Quantification

Total RNA was extracted from 100 µl of entire blood using the Norgen Bioteks "Total RNA Purification" kit

according to the manufacturer's procedure with slight alterations, for example the samples once homogenization incubated on ice bath for 30 minutes and then centrifuged at 4℃, speed 13 000 g for 5 minutes, and 200 µl volume of 70% ethanol used for precipitation. 350 µL of lysis buffer added to the mixture followed by 15 seconds of vortex. 600 µl of the sample has been transferred to spin column with collection tube followed by centrifuge for one minute at 6000RPM. In case if lysate did not pass through the column further centrifuge has been applied at fourteen-thousand RPM for two minutes. Twenty-five µL of elution buffer was added to the spin column on the silica membrane to progress RNA yield. Then centrifuged of two-thousand RPM for two minutes has been applied to the column. Twenty-five µL of the remaining elution buffer was added to the column and centrifuged at fourteen thousand RPM for one minute. The purified total mixture transferred was stored at -80℃ until assayed.

3.1.3 Gel Electrophoresis

Throughout this study 1.5 % of agarose gel was used. By prepared 1.5 g of agarose which added to 100 mL of 1X TAE buffer. Followed by heating it at 80℃ by using microwave to melt the mixture and then allowed to cool the mixture till 60℃. 3 µL of ethidium bromide has been added to the solution till its solidify. The gel was run at 100 volts for twenty-five minutes.

3.1.4 Revers Transcription Reaction

Reverse transcription reactions have performed in a volume of 15 µl, using the (New England Biolabs) Reverse Transcription Kit. Every reaction contained 100 ng of entire RNA, 6 µl TaqMan MicroRNA Assay RT Primer Pool (containing PER1, PER3, MKNK2 and CABRIN1), 0.2 µl of 100mMdNTPs, 3 µl of MultiScribe Reverse transcriptase enzyme (50 U/µL), 1.5 µl of reverse transcription buffer (10x) and 0.19 µl of RNase Inhibitor (20 U/µL). The RT primer pool was prepared according to the manufacturer's guide with reference to custom primer pool preparation with the exception of using nuclease-free water instead of 1x TE buffer. Negative controls contained distilled water as an alternative of RNA was included. RT reactions was performed on MJ Research Thermal Cycler PTC-200 with the following program: 16℃ for 30 minutes, 42℃ for 30 minutes, 85℃ for 5 minutes and 4℃ on hold. Samples kept at -20℃ until assayed.

3.1.5 RT-Qpcr REACTION

PER1, MKNK1 CAPRIN1 and PER3 expression levels have tested in our samples. The RT reaction contained 0.5 µl of suitable TaqMan MicroRNA Assay (Applied Biosystems), 2 µl of two times diluted RT product, 5µl of TaqMan Fast Universal PCR Master Mix (2x) NoAmpErase UNG (Applied Biosystems) and 2.5 µl of nuclease-free water in total volume of 10 µl. No-template controls (NTC) with distilled water instead of RT reaction product included. All reactions ruined in triplicate in 384-well plate format on Roche LightCycler 480 (Roche Diagnostics, Mannheim, Germany). The program consisted of a 10 minutes' pre-incubation step in 1 cycle of 95℃ for 10 min, 45 cycles of :95℃ 10 sec, 60℃ 10 sec, 72℃ 10sec and 1 melting curve step from 65℃ to 97℃.

4. Results

4.1 Rna Extraction

After running the 2.0% agarose gel and using the UV/light in order to check the appearance of the RNA in our samples as mentioned in below figure. Band number one represent the genomic DNA. In the main while band number 2 is rRNA corresponding 28S. In addition to number 3 is 18S. After that spectrophotometer has been processed to confirm the RNA quality by using the ratio of the wavelength 260 and 280 which was most of the time approximately equal 2.

4.2 First Strand Cdna Synthesis

It was Confirmed the successful of converting the RNA to cDNA process by run a 2.0% agarose gel and appearing of cDNA bands as showed in figure number 5

At the end of PCR reaction, Ct values for every and each primer are available.

4.3 Pcr Optimization

Many trailed-on PCR were done in order to optimize the primers but the conditions were not workable to have the amplification. So that optimization step was skipped and moved to RT-PCR.

4.4 RT-PCR

At the end of PCR reaction, Ct values for every and each primer are available as well as the amplification curves and melt peak which show the absence of none specific binding of the primers which confirm the presence of the RNA expression.

5. Statistical Analysis

Gene expression data were analyzed with delta cycle threshold, non-liner regression, correlation and normalize method using GAPDH as the reference gene following simple Δ Ct method, where Δ Ct = Ct target – Ct reference. By using Graphed Prism. Starting with MKNK2 profile, the maximum expression is 31.51 was at 12. With comparing (K. Lech and his colleagues/ Forensic Science International) they found the maximum expression is between 15:30h and 17:00h.

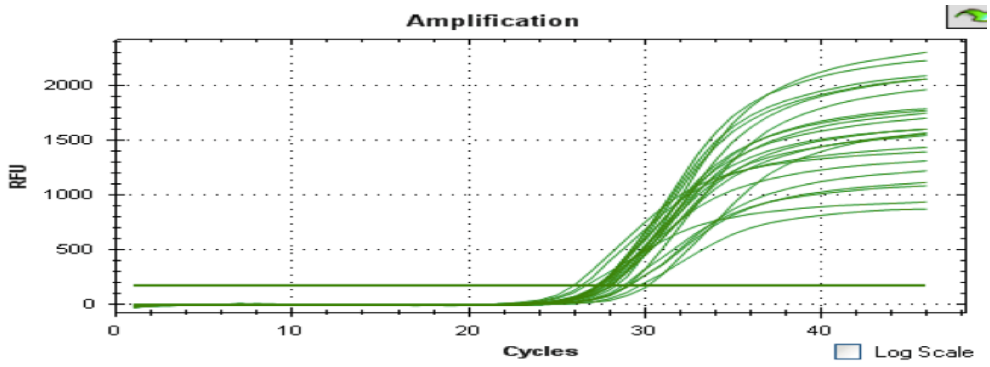

Figure 2: Quantification curve for PER 1 marker.

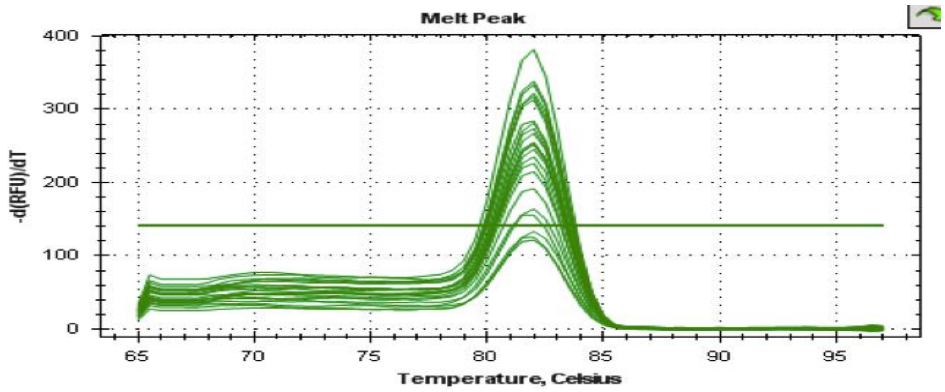

Figure 3: Melt curve for PER 1 marker.

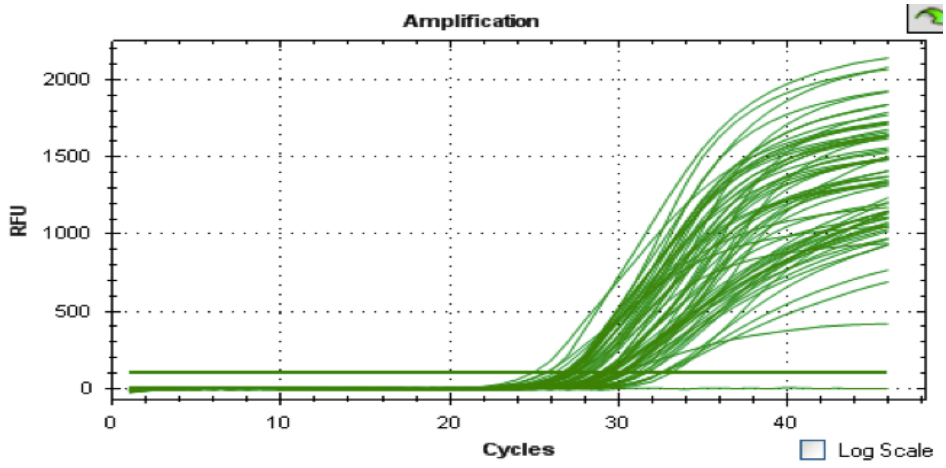

Figure 4: Quantification curve for MKNK2, CAPRIN1 & PER3 marker.

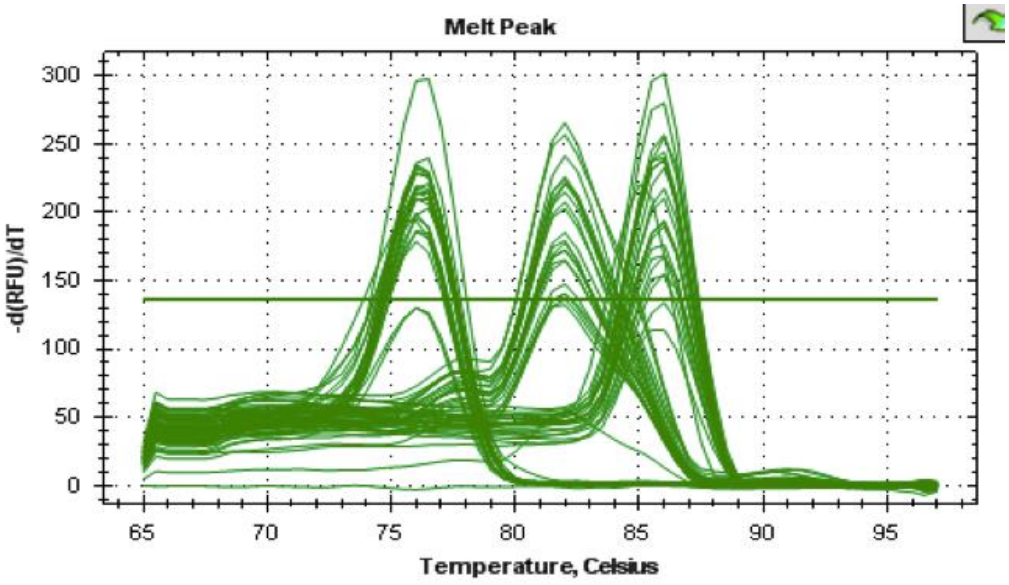

Figure 5: Melt curve for MKNK2, CAPRIN1 & PER3 marker.

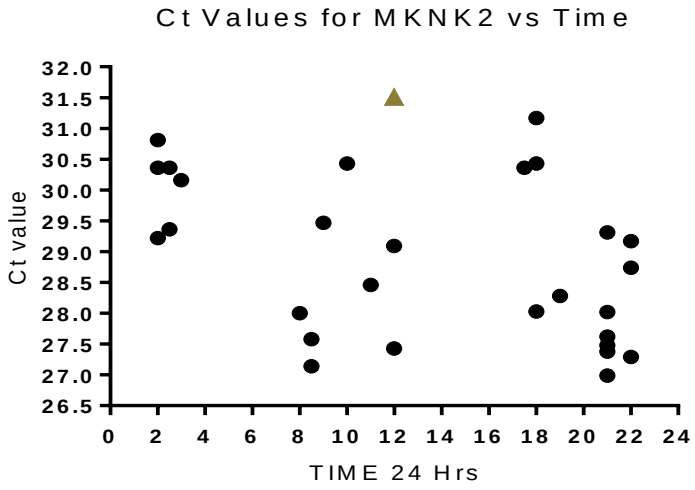

Figure 6: CT Values for MKNK2 VS Time

Table 1: Prediction model was established based on logarithm logistic regression in order to confirm the profile expression for MKNK2 was significant as following

Correlation coefficient value	-0.3980
95% confidence interval	-0.6673 to -0.03683
R square value	0.1584
P (two-tailed)	0.0325
alpha	0.05

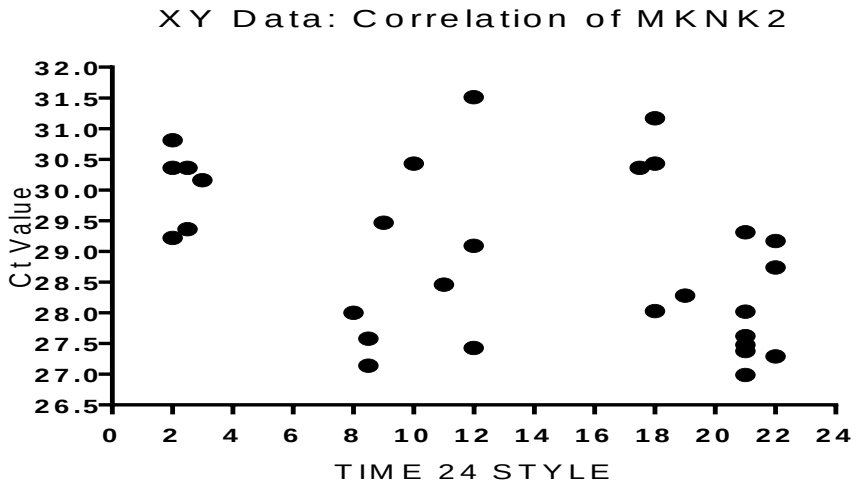

Figure 7: Correlation of MKNK2

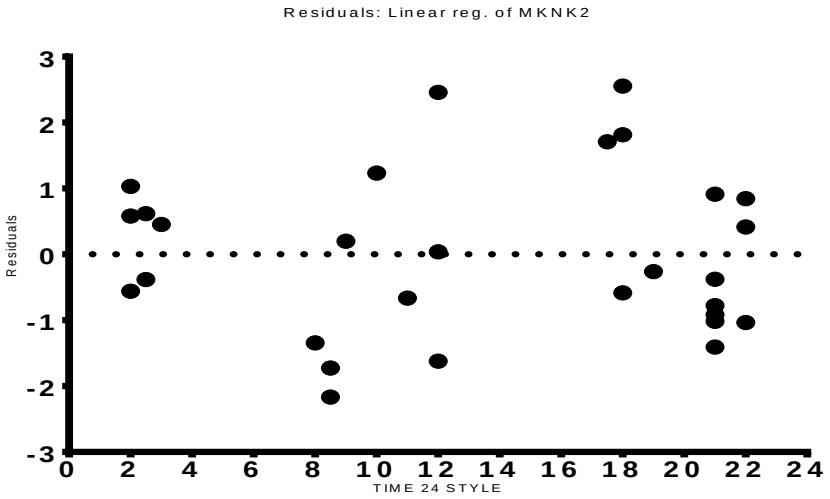

Figure 8: Residuals of MKNK2

Looking at the residual part, we can say its random all over the place and there is not real identification of specific pattern which mean the residual can define. The meaning of residual is the predicate data – observed value. In order to have the value of predicate Ct value. The equation of non-liner has been evaluated as y = -0.806ln(x) + 30.935 in order to evaluate the SD which equal 110696.44. The maximum expression is 33.21was at 18. With comparing (K. Lech and his colleagues / Forensic Science International) they found the maximum expression at 22.

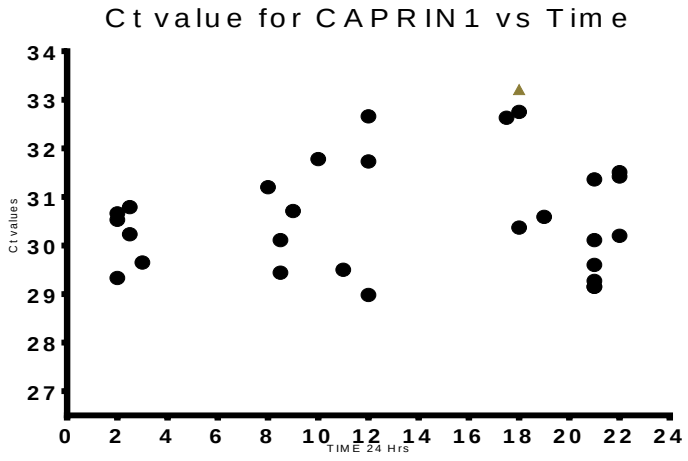

Figure 9: CT Values for CAPRIN 1 VS Time

Table 2: Prediction model was established based on logarithm logistic regression in order to confirm the profile expression for CAPRIN1 was NOT-significant as following

Correlation coefficient value	0.1209
95% confidence interval	-0.2571 to 0.4668

R square value	0.01462
P (two-tailed)	0.5321
alpha	0.05

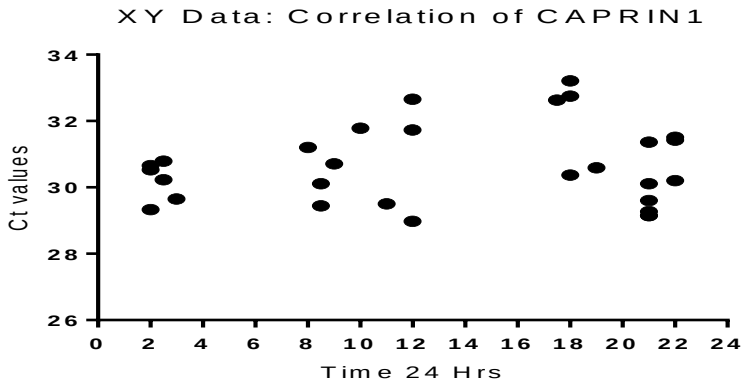

Figure 10: Correlation of CAPRIN 1

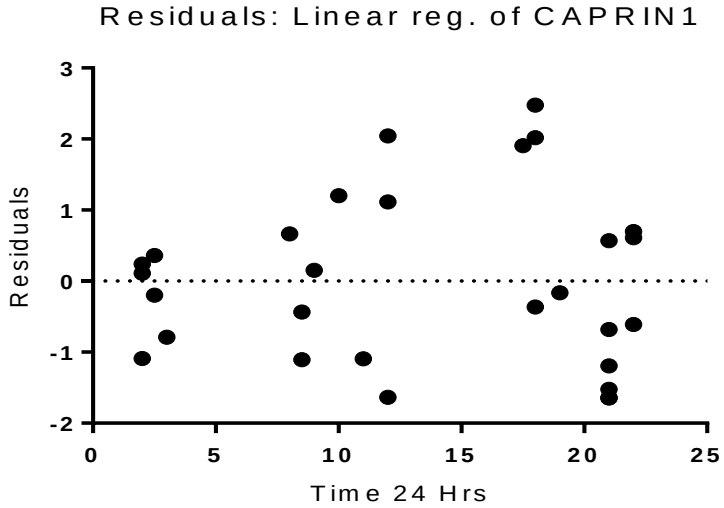

Figure 11: Residuals of CAPRIN 1

Looking at the residual part, we can say its random all over the place and there is not real identification of specific pattern which mean the residual can define. The meaning of residual is the predicate data – observed value. In order to have the value of predicate Ct value. The equation of non-liner has been evaluated as $y = 0.0349\ln(x) + 30.556$ in order to evaluate the SD which equal 108361.83. The maximum expression is 30.37 was at 4. With comparing (K. Lech and his colleagues / Forensic Science International) they found the maximum expression at 22.

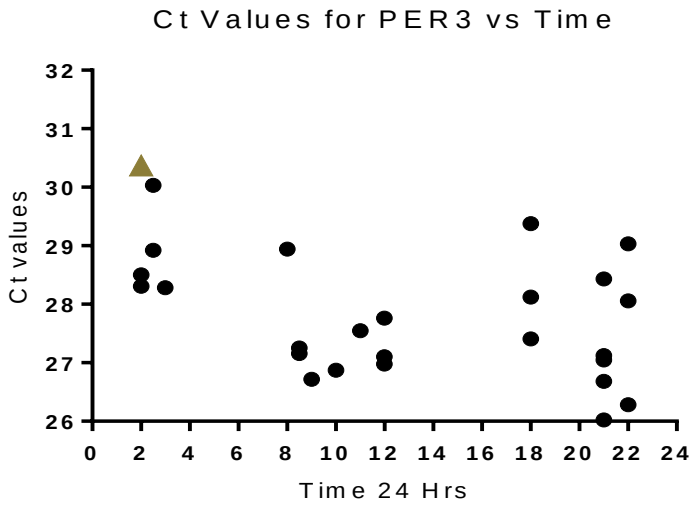

Figure 12: CT Values for PER 3 VS Time

Table 3: Prediction model was established based on logarithm logistic regression in order to confirm the profile expression for PER3 was significant as following

Correlation coefficient value	-0.5062
95% confidence interval	-0.7362 to -0.1714
R square value	0.2562
P (two-tailed)	0.0051
alpha	0.05

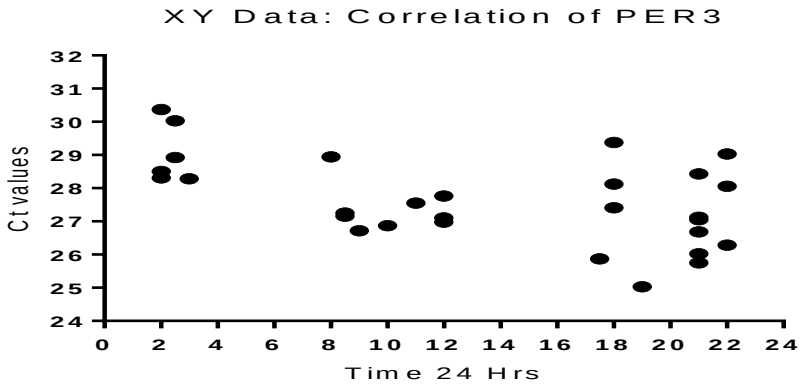

Figure 13: Correlation of PER 3

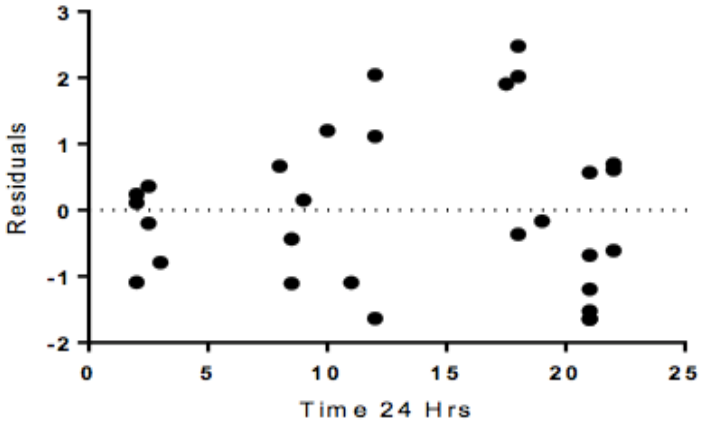

Figure 14: Residuals of PER 3

Looking at the residual part, we can say its random all over the place and there is not real identification of specific pattern which mean the residual can define. The meaning of residual is the predicate data – observed value. In order to have the value of predicate Ct value. The equation of non-liner has been evaluated as y = -0.865ln(x) + 29.743 in order to evaluate the SD which equal 102293.71. The maximum expression is 30.18 was at 2.5. With comparing (K. Lech and his colleagues / Forensic Science International) they found the maximum expression between at 8hr and 10hr.

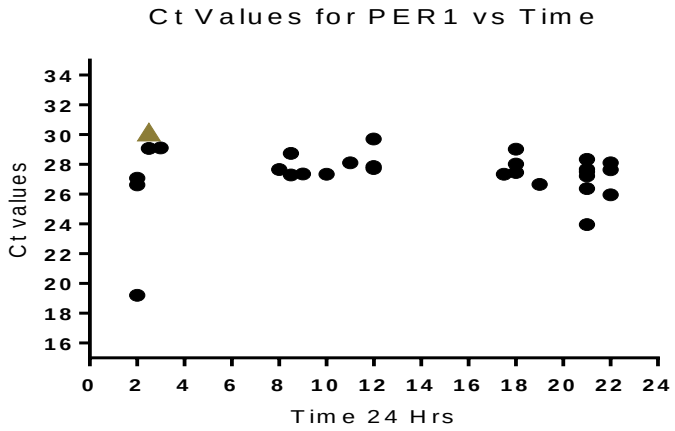

Figure 15: CT Values for PER 1 VS Time

Table 4: Prediction model was established based on logarithm logistic regression in order to confirm the profile expression for PER1 was NOT-significant as following

Correlation coefficient value	0.008056
95% confidence interval	-0.3596 to 0.3735
R square value	6.489e-005
P (two-tailed)	0.9669
alpha	0.05

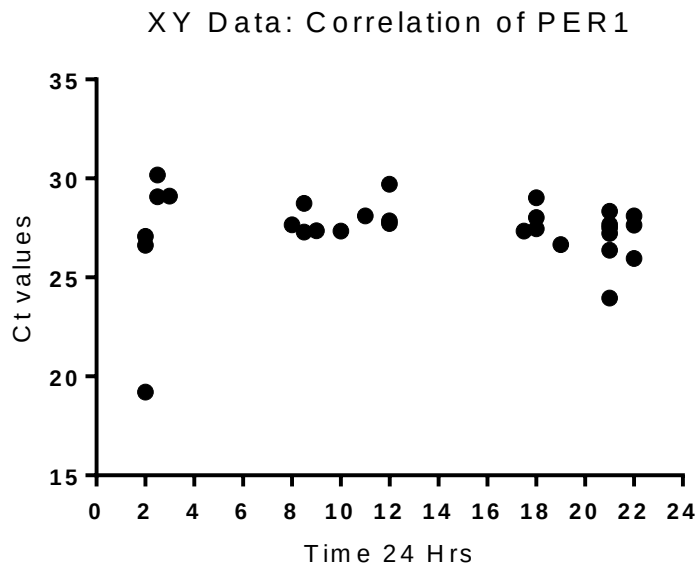

Figure 16: Correlation of PER 1

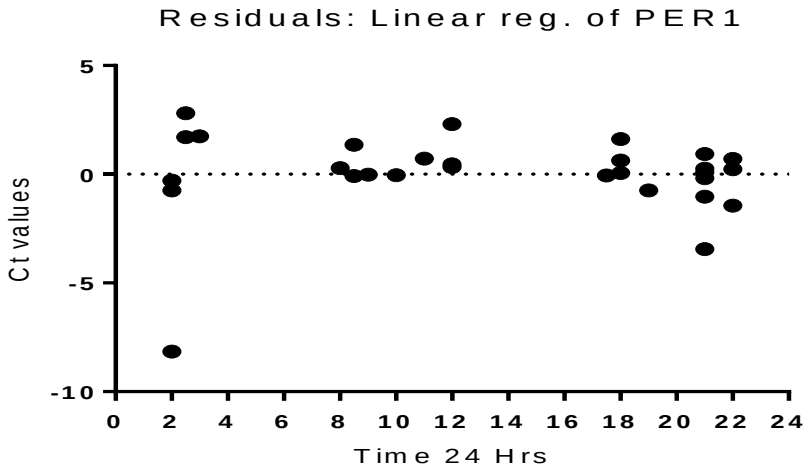

Figure 17: Residuals of PER 1

Looking at the residual part, we can say its random all over the place and there is not real identification of specific pattern which mean the residual can define. The meaning of residual is the predicate data – observed value. In order to have the value of predicate Ct value. The equation of non-liner has been evaluated as $y = 0.9165\ln(x) + 25.14$ in order to evaluate the SD which equal 73754.43.

6. Discussion

This research aimed to evaluated whether the selected genes are expressed in a rhythmic style in human blood and, if so, to differentiate the genes that exhibit daily 24-h rhythmicity, check their stability and significantly rhythmic. After long research, 4 core clock genes, MKNK2, CAPRIN1, PER3 and PER1, expressions have been

analyzed to assess their circadian rhythmicity as observed in (K. Lech and his colleagues Forensic Science International,2016). Results of the current study have showed relatives differences compared to the finding reported for the same mRNA markers. Among all the investigated markers, MKNK2 has shown the nearest compared to the previous study with rhythmic difference is approximately 3 hours. Since it shows the max expression at 12:00 PM compared to 15:30 PM in the previous study. In the current study the maximum expression of CAPRIN1 was at 18:00 PM whereas, the pervious study has reported the maximum expression for the marker was at 22:00 PM. So, the rhythmic difference is approximately four hours. In (K. Lech and his colleagues Forensic Science International,2016) have reported two peaks for the maximum expression for the PER1 and whereas in our study was rhythmic expressed at 2.30 AM. So, the rhythmic difference is approximately from 6-7 hours. This might happen due to entrain the peripheral clocks, causing to uncoupling between the central and peripheral oscillators in mammals and even to phase shifts and composition or timing of meals might affect the expression of metabolism-related genes, as timing and meal composition influence gene expression. In the current study the maximum expression of PER3 was 4:00 AM however, the pervious study has reported the maximum expression was at 10:00 PM. The different might be attributed to some other internal and external influence on circadian biomarker which have been previously reported. MKNK2, CAPRIN1 and PER1 exhibited significant time-of-day variation in expression during the second and third, fourth and first 24 hr respectively of the study. As mentioned before Circadian rhythms can be characterized by three inherent properties. First of all, they are self-sustaining or free-running, persisting in the absence of any external time cues (i.e. light/dark cycle) with a period of approximately 24 h. Secondly, they are susceptible to resetting (entrainment), by external time cues (zeitgebers) such as the daily light/dark cycle, temperature or food. Lastly, the circadian rhythms are temperature compensated, meaning that their period length is constant over a wide range of physiologically relevant temperatures. All those features contribute to a robust, yet susceptible to fine-tuning, circadian system. So that we don't have to centralized and standardized the concept of using the circadian biomarker and take into consideration the above factors which alter in the sensibility and the stability of them. There are many reasons to explain the difference between this study results and the (K. Lech and his colleagues / Forensic Science International). [6] Circadian oscillations are reflected in various biological processes like hormone secretion, metabolic reactions, behavior, and many others [10–13]. Some factors affect the two main circadian hormones which are melatonin and cortisol which has a direct influence on circadian biomarker, and due to the limited time resolution, they provided when being applied to trace deposition timing. So, consider the difference between the circumstances of (K. Lech and his colleagues Forensic Science International,2016) which done in Netherlands and our research which done in UAE. For example, the difference of, intensity of the light, temperature and duration of the day.

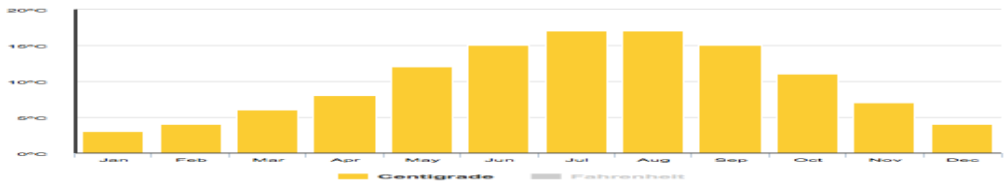

Figure 18: Average Temperature in Netherlands http://www.holiday-weather.com/amsterdam/averages/

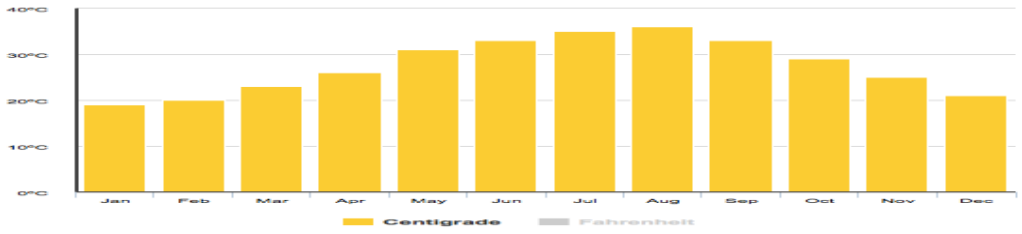

Figure 19: Average Temperature in UAE http://www.holiday-weather.com/dubai/averages/

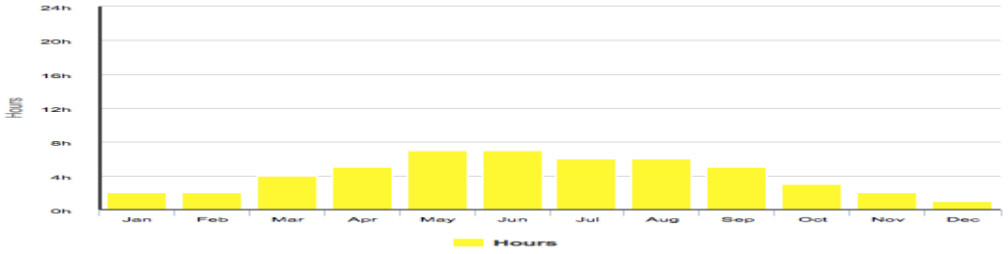

Figure 20: Average Daily Sunshine Hours in Netherlands http://www.holiday weather.com/amsterdam/averages/

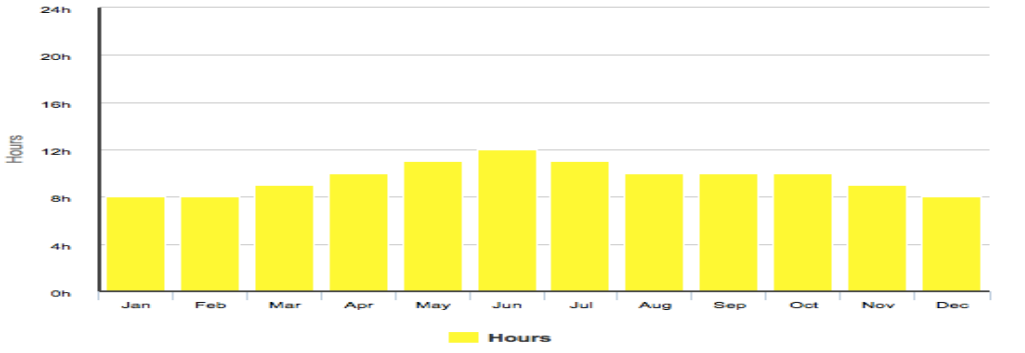

Figure 21: Average Daily Sunshine Hours in UAE http://www.holiday-weather.com/dubai/averages/

For example, melatonin secretion is inhibited by exposure to light, in a dose–dependent manner [17,18]. In normal subjects' melatonin suppression starts between 200–400 lux (equivalent to ordinary fluorescent light), however upon light removal, melatonin concentration returns to normal night time levels [17]. In another study, it has been shown that acute suppression of melatonin secretion occurs after exposure to intensive light (600 lux or higher) for an hour [17,18]. Furthermore, a disruption in melatonin's circadian patter has been noted in subjects suffering from mental disorders such as major depressive disorder [19]. Cortisol levels have been shown to be disrupted in individuals suffering from addiction, chronic stress, or posttraumatic stress disorder (PTSD) [20–22]. So, different circumstances should be under consideration in order to adopt and simulate the condition in different crime scene.

7. Conclusion

In this study, we investigated whether mRNA provides a suitable resource for establishing biomarkers to estimate blood deposition time. We introduced a prediction model comprising with previous data from other research

paper. To achieve a more detailed level of time category prediction than revealed here, additional rhythmic biomarkers with different peak times will be needed. Future studies should focus on identifying them, and eventually incorporating them together with the markers used here to develop a final prediction model. Additional testing in other forensically relevant tissues, such as saliva, semen, skin, vaginal secretion, and menstrual blood are required. As it would also be advantageous in determining whether the mRNA markers proposed here are also informative for time of death. Another crucial aspect that shall be tested carefully in future studies is the time-wise stability of the proposed mRNA markers. Since blood stains are found at various crime scenes, they are exposed to a multitude of variable conditions, i.e. high/low humidity, drought, temperature changes, but are also located on different types of surfaces. All these factors can possibly influence the stability of the mRNA, which should be tested for the specific mRNA markers proposed here. One more thing, Future forensic application must combine circadian hormone with mRNA expression to enhanced the time prediction accuracy relative. Moreover, our work provides and compare new leads for future studies on time of death investigation using the significantly rhythmic mRNA markers established here, which represents a second aspect of forensic time estimation in need of improved biomarkers, methodology, and technology. Further researches might discover out if miRNA markers with significant circadian expression patterns might be acknowledged, to achieve reliable and narrow time predictions, and how convenient they might be for forensic evidence deposition timing. Because in all those previous studies only a small number of biomarkers were tested, this ultimately limited the precision and significance of the obtained time estimates [6,19].

List of Abbreviation

Table 5

RNA	Ribonucleic acid
mRNA	Messenger Ribonucleic acid
DNA	Deoxyribonucleic acid
STR	Short tandem repeat
SCN	Suprachiasmatic nuclei
bHLH-PAS	Basic helix-loop-helix
Per	Period
Cry	Cryptochrome
ipRGCs	Intrinsically photosensitive retinal ganglion cells
RHT	Retinohypothalamic
PMI	Post mortem interval
ELISA	Enzyme-linked immunosorbent assay
SOCO	Scene of Crime Officer
UV	Ultraviolet
Hb	De-oxyhemoglobin
HbO2	Oxyhemoglobin
met-Hb	Methemoglobin
RBC	Red blood cells

WBC	White blood cells
(-OH)	Hydroxyl
tRNA	Transfer Ribonucleic acid
rRNA	Ribosomal Ribonucleic acid
PCR	Polymerase chain reaction
RT-PCR	Real time polymerase chain reaction
miRNAs	Micro Ribonucleic acid
CCGs	Clock-controlled genes
CR	Constant routine
S/SD	Sleep/sleep deprivation

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