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INTRAUTERINE FETAL DEATH: A FORENSIC-PATHOLOGY STUDY ABOUT THE ESTIMATION OF TIME OF DEATH

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1.

INTRODUCTION AND PURPOSE

In forensic pathology practice is of fundamental importance the diagnosis of time of death. If such an investigation is often complex with a well-preserved corpse, it is even more difficult in the case of a fetus dead in-utero affected by macerative post-mortal phenomena.

In such circumstances, clinical data can certainly prove to be helpful to the medical examiner; more often, however, it appears that the last ultrasound control with demonstration of fetal heartbeat has been performed days or even weeks before the stillbirth relief. In addition, the period of fetal movements absence reported by the mother can not be considered by itself as demonstrative of the time of death, in the absence of the necessary requirements of objectivity, scientific validity and relevance which are essential in the conduction of an investigation that should ensure high levels of technical-scientific evidence in a judicial context.

The thanatological survey acquires further importance and complexity if it is taken into consideration that the medico-legal autopsy of a fetus dead in-utero is often a technical judiciary assessment into an investigation on suspicion of professional obstetrical and gynecological malpractice. Among the questions commonly placed by the Judicial Authority is the identification of the time of death, and this point often extends any subsequent trial phase of the process: clearly, different responsibilities profiles are delineated in the case of a fetal death followed by a prolonged retention

time in the uterus (and only then comes the observation of the obstetrician) rather than a fetal death few hours before the expulsion time, which occurred when the woman was already hospitalized within a hospital structure.

It is clear that in such cases the diagnosis of the time of death is inextricably based on examination of the fetus and the placenta: this is often the only objective technical assessment with the necessary scientific criteria and objectivity.

The pathological and obstetrical literature is rather thin about the subject, recording only sporadic report case studies and even more rare experimental studies that focus on macro- and microscopic characteristics of fetus and placenta (skin maceration, nuclear basophilia, placental abnormalities, etc.) which currently represent just over a slow evolution (when not just an explicit repetition) of parameters and assessments dating back to the '60s and '70s, based solely on empirical data of experts in fetus and placenta pathology.

These criteria currently available in the literature, almost never validated and without a solid experimental basis, are inappropriate or useless in clinical practice, where the medical examiner often experience a complete mismatch between morphological and clinical data: the overall assessment is then allowed to base upon the experience of the individual rather than on scientific criteria.

The present study therefore arises from the necessity to first assess the actual validity of the thanatological criteria proposed in the literature through a retrospective review of a selected population, and thereafter will develop an experimental prospective phase with the purpose of updating the "old criteriology" proposing a "new criteriology" which can allows a better and more detailed post-mortem analysis

of the phenomena of the fetus died in-utero, especially where the above criteria will be shown more incomplete and misleading, transforming those who today are considered to be “confounding factors” in real “correction factors” of judgment.

2.

ASSESSMENT CRITERIA OF FETAL THANATOLOGY

In most fetal deaths the precise moment of death and the duration of the intrauterine retention postmortem period are not known, especially when death precedes labor or hospitalization.

The mother often reports no fetal movements for a few days, but this information, although valid to put a clinical suspicion and proceed with the investigation of the case, can not (and should not!) be taken as the main reference for the dating of the fetal death (among others: Heazell and Frøen 2008). As already noted in the Introduction, the absence of fetal movements perceived by the mother lacks the necessary requirements of objectivity, scientific validity and relevance that are essential in the conduct of a judicial investigation. During a forensic investigation for suspected professional malpractice for failure to ensure the survival of the fetus, the thanatological judgment is preliminary and essential to ascertain whether in such situations, regardless of the physicians work, the fetus was actually alive at the time of the allegations or was already dead, with consequent absence of any hypothetical causal link between the alleged negligent conduct and the death of the fetus (Umani Ronchi et al. 2002). In these cases, therefore, the thanatological judgment based upon fetus maceration is fairly complex and difficult to resolve, entrusted primarily to the macroscopic evaluation of the fetus and a few histological indicators (Fulcheri et al. 2006; Umani Ronchi et al. 2002).

After his death, the fetus may still remain inside the uterus for a variable period of time, during which cutaneous and visceral changes called "maceration" develop. The maceration is a phenomenon that, strictly speaking, is manifested only in stillbirth retained in the uterus, with ovular membranes intact, when there is yet sterile amniotic fluid. In fact, it is only realized when the body is not contaminated by putrefactive germs and, regardless of their action, evolves essentially for autolysis associated with a particular tissue imbibition. The unique environmental factor is decisive for the evolution of the macerative process. In the event of reabsorption of amniotic fluid, the died fetus retained in the uterine cavity may be subject to additional and different transformational processes in relation to changing environmental conditions, such as mummification and the eventual calcification with the formation of the so-called "lithopedion", or putrefaction if the uterus is contaminated by germs (Philips et al. 2006; Gerin et al. 1997; Perper 2006; Umani Ronchi et al. 2002).

The first observational studies about fetus maceration appear in the scientific literature from the first half of the nineteenth century. The research in this field were initially undertaken in the context of forensic science, with the intent to clarify the differences between in-utero maceration and the simple water immersion, especially with the aim of diversifying fetal intra-uterine autolytic changes from common post-mortem alterations in case of infanticide.

One of the first studies in the literature is that of the well-known French coroner Orfila, who recorded in 1828 a series of experimental observations on the alterations of dead fetuses experimentally exposed at various temperatures in different

media (atmospheric air, gas, stagnant water and electricity), proving that different parts of the body do not degenerate with the same speed and that the decomposition occurs faster in current water rather than stagnant (Orfila considered the same way as stagnant water also the amniotic fluid in which the fetus was plunged). After the studies of Orfila, in 1867 followed one of the most extensive monographs on post-mortem changes of the embryo and fetus by Lempereur, who concentrated his own observations on the macerative phenomena, providing perhaps the first chronological classification of the various macerative phases. The next forensic contribution was made by Sentex in 1869: he described the changes observable with naked eye at different macerative periods of the fetus to differentiate macroscopic appearance of the macerated fetus from that of the fetus underwent post-mortem immersion, easily distinguishable according to the author. Sentex was also the first to identify as a pathognomonic sign of fetal maceration the pink color of the eye structures. In 1880 another contribution was exposed by Hourlier during a presentation at the University of Paris. The author focused his attention on the differences between in-utero maceration and water submersion, concluding that the latter produces a hard tissue edema due to imbibition, and that the color of the skin is pale rather than rosy; he put the attention on the fact that intracavitary blood flow does not usually take place in bodies submerged in water, differently from what happens in in-utero maceration (from Thomson 1927).

In 1922 Spalding described the overlap of skull bones (visible with X-ray survey) as a pathognomonic sign of intra-uterine death, which manifests itself with the

progression of macerative phenomena; today we still refer to the "Spalding sign" as ultrasound and radiological sign of intrauterine fetal death.

Again in 1922 Strachan published a study of 24 cases of macerated fetuses expelled between 24 and 36 weeks of gestation, with the intent to seek any possible correlations between maternal diseases (especially syphilis) and the degree of fetal maceration: even if his research showed up with nothing, Strachan offered one of the first macro-/microscopic descriptions of the different degrees of fetal skin, visceral, bone, and placental maceration; he also drew conclusions that are entirely up to date even today, almost 90 years after its publication, that are worth repeating here in the essential points: "*It would appear that maceration is brought about by soddening of the unprotected foetal skin by the liquor amnii, accompanied by an autolysis of the internal organs by enzyme action. […] Maceration is the result of retention of the dead foetus* in utero*, whatever be the cause of death. […] In the investigation of any macerated foetus it is essential to have the placenta for examination also. […] The microscopic findings indicate a process of autolysis of the constituent cells of the various organs with oedema of the interstitial tissue and extrusion of red blood cells*". In his work, Strachan had no claim to indicate a precise chronology of the various macerative stages, but he offered a full, accurate and effective description of fetal maceration, although rudimentary. The first change observed after death was the disappearance of the vernix caseosa, after which the skin absorbe liquid and the skin is easily detached from the underlying dermis, leaving exposed a layer of deep red color skin; in the later stages this phenomenon extends to various parts of the body ("*the skin looks as though eroded in patches*"), especially the abdomen, scrotum and limbs, to affect the entire skin surface. Simultaneously, also internal organs undergo a process of progressive lysis for the

action of enzymes, precociously evident in skeletal muscle, which causes a decrease in organs consistency, and a diffuse brown discoloration of the parenchyma due to the effect of hemolysis, as well as multiple hemolytic flows in various serous cavities, and joint laxity due to the lysis of ligaments. With regard to the placenta, which is essential for the identification of the cause of death, the common sign of maceration is a greenish discoloration and a viscous appearance of the amniotic surface; the umbilical cord initially appears flaccid, then soaked, edematous and reddish in color. Finally, in microscopic examination of the various organs, the first observable change is a decrease in the stainability of the nuclei; subsequently also the cytoplasm is colored irregularly and margins become undefined.

It deserves to be pointed out that despite the efforts of some authors and the identification of additional few markers of maceration, the description of macro-and microscopic findings did not change, nor is it merely changed the observational and descriptive approach to the macerated fetus on which it is still expressed the thanatological opinion. For example, still in 2005 Maroun and Graem, in their study on the comparison of body and organ weights between macerated and non macerated fetuses, subdivide the macerated fetuses population using purely observational criteria, according to the macroscopic external appearance: "*Mild maceration was defined as bullae or skin slipping on extremities or small parts of the face or trunk. Moderate maceration was defined as extensive skin slipping and reddish discoloration of the skin and umbilicus. Marked maceration was defined as brownish, tan, or yellowish skin discoloration, overlapping cranial bones, loose joints, and/or mummification*".

THE FETUS

The recommendation to proceed to the evaluation of the maceration degree during fetal autopsy in order to make a judgment on the time of death and the period of intra-uterine retention is present in any protocol or guideline regarding diagnostic and post-mortem investigations in case of intra-uterine death (among others: Busuttil and Keeling 2009; Di Maio and Di Maio 2001; Dimmick and Kalousek 1992; Magee 2001; Moore 2001; Ochs et al. 1988; Saukko and Knight 2004; Wainwright 2006). Moreover, from a forensic point of view, in the case of the discovery of a fetus corpse that come to the attention of the Judicial Authority with a suspicion of infanticide, the presence of cutaneous macerative signs can be considered as definitive evidence that fetal death occurred before birth and the fetus itself remained held in-utero for a variable period of time; in these cases it is necessary to distinguish preliminary signs of maceration from putrefactive phenomena that may be occurred after the expulsion (Busuttil and Keeling 2009; Di Maio and Di Maio 2001; Saukko and Knight 2004).

Unfortunately, for many years pathologists have been content with a diagnosis of "macerated fetus"; anyway, from a macerated fetus we can obtain useful information and also the non-diagnosis of "macerated fetus" may become appropriate if you are able to quantify the process of maceration and give explanation of the facts (Fulcheri et al. 2006; Genest 1995; Moore 2001). Today it is repeatedly stressed that the autopsy of a fetus can be conducted with profitable results despite of the presence of maceration (Magee 2001, Moore 2001). The common volume and weight reduction of the body and individual organs (especially liver, thymus and spleen) observable with the advance of maceration, has been the subject of numerous studies and to date all of

these parameters are standardized with reference to measurements and weights of non macerated fetuses (Maroun and Graem 2005).

Several perinatal pathologists have tried to define useful criteria to determine, at least approximately, the time of fetal death in relation to the period of intrauterine retention of the fetus after death, mainly based on the degree of skin maceration and organs autolysis. During the nineteenth century, as we have seen, studies on fetal maceration were purely descriptive, offering little from a chronological point of view but generic directions of transformations whose appearance could be observed sooner or later than others.

One of the first experimental studies, although empirical and based on personal observation not subject to scientific validation, was due to Thomson in 1927, with the aim of defining a timeline of fetal macerative phenomena. The author divided fetuses into three stages of maceration compared to intrauterine retention time, based on the clinical history reported by the mother: stage I (1 to 10 days), stage II (10 to 40 days) and stage III (more than 40 days). For each stage he provided an extensive description of the macro- and microscopic characteristics of the fetus, the fetal organs and the placenta. Unfortunately, despite the very accurate and thorough descriptions, Thomson work is affected by the decision to include fetuses in one of the three stages based solely on mother's reports, which has in fact led to an apparent abnormal lengthening of the time of maceration: for example, in stage II is described the presence of an initial skin desquamation and few dermo-epidermal bubbles still intact. Stage II (10-40 days) could be more appropriately framed today in "within 48 hours".

Another early study of the chronological fetal macerative processes reported in scientific literature comes from the Italian medico-legal work done in the '30s by Perrando (Perrando 1935, later taken by: Perrando and Macaggi 1940). Although dated and empirical, these indications are still the most analytical in the Italian medico-legal literature, and probably in international literature considering the years in which they were published. The aim of Perrando work, already for those years, was far beyond the mere pathological interest, as pointed out by the author himself: "*stages of this macerative process are important to know in order to draw criteria of judgment on the time of death of the fetus in-utero in relation to issues of potential criminal malpractice related to pregnant women… or use of abortive means*". It seems appropriate to quote here an overview of the observations of Perrando and Macaggi, not only for historical curiosity, but also to highlight the descriptive rigor (Table 1).

Another important experimental study of fetal histology was undertaken in 1964 by Shanklin, with the aim to correct the chronological sequential pattern proposed by Thomson in 1927. Shanklin examined 53 human fetuses died in utero, with known recorded timeframes during hospitalization: membranes rupture, duration of labor, the last fetal heart auscultation, the time of the absence of fetal heart auscultation, the interval between the expulsion and the autopsy. For each fetus, Shanklin analyzed 46 histological features of 4 organs (skin, lung, liver and kidney) and described them as 0, trace, 1+, 2+, 3+ or 4+, without defining the precise method of gradation; the purpose of the study was to validate on a human population the histological criteria for the determination of the fetus time of death that had previously developed using an animal model. Table 2 shows the main results from the animal model (Shanklin et al. 1964).

	Up to 3rd day	Up to 6th day	Up to 9th day	Up to 12th day	Up to 15th day	Beyond 20th day
Shape	almost normal	rather pendant	great flaccidity	same	same	same
Copper color	limited to face or neck	widespread but slightly accentuated	accentuated with blackish color of the abdomen	blackish color widespread to the thorax	same	begins the disintegration of all the tissues and organs
Skin	still intact	softened	break up of the stratum corneum	epidermal exfoliation widespread to the head	same	
Serous cavity and blood infiltration	missing except the base of umbilical cord	begin cavity flows	significant flows and infiltrations	flows that infiltrate organs	infiltration of all tissues	
Brain	soft but sustained	crushable	tending to pultaceous	pultaceous	reduced to rosy fluid	
Lungs	fetal and dark	same	tending to purple	color same as other tissues	friable	
Liver	almost normal	more dark and friable	becomes yellowish	even more yellowish at the surface, and blackish internally	more friable, dark-yellowish, with detaching capsule	
Ocular fluids	normal	not yet dyed	rosy dyed, except the lens	blood dyed widespread and intense	dyed also the sclera	
Cellular elements	increase in stainability of the nuclei	alteration of cell contours, lower colors affinity	fusion of protoplasm and fragmentation of nuclei	uniform masses and debris in which there are nuclear granulations	reduction to inform debris	

Table 1. *Synoptic table of the process of fetal maceration, according to Perrando 1935 and Perrando and Macaggi 1940.*

Shanklin's estimation of time of fetal death resulted in agreement with clinical data in 50 cases out of 53, two fetuses showed higher estimates than the probable period from the death and one fetus a lower estimate. For one of these cases Shanklin hypothesized that the presence of pathological conditions may have had an accelerating effect on the macerative phenomena (the fetus was suffering from erythroblastosis, anemia and hydrops). For the other two cases Shanklin "blamed" the

inconsistency and the fallacy of the auscultation data. In fact, the main weakness of the study concerns the auscultation sounds, which should have be the main and best selected criteria of the study: the time of the fetal heartbeat survey is missing or doubtful in 19 cases, while the time of the absence heartbeat survey is missing or doubt in 7 cases. Shanklin itself admitted that in only 3 cases the available clinical data (with not excessively spaced interval) could be properly used for the purpose of experimental validation of the criteria. In the remaining cases the author highlighted a low level of adequacy of clinical documentation with reference to the time of fetal death.

Organ	**Time** *(*)*	**Histological characteristics**
Skin	90 min.	perinuclear halo formation in epidermis cells
	90 min.	leakage of red blood cells from vessels and absence of red blood cells in the dermis
	9 h	formation of bubbles between the epidermis and dermis
	12 h	formation of dermo-epidermal vesicles
	12 h	disappearance of perinuclear halos in epidermis cells
	48 h	rupture of dermo-epidermal vesicles
	96 h	initial decrease in basophilia
	150 h	present only a minimal basophilia
Kidney	90 min.	the nuclei in Bellini ducts become irregular
	6-12 h	cells of Bellini ducts are separated from the basal membrane
	30 h	detachment of endothelial cells from renal vessels
	48 h	persistence of basophilia in the glomerular zone
	150 h	complete loss of basophilia
Liver	90 min.	loss of cohesion between the liver structures
	9-24 h	progressive loss of red blood cells
	12-15 h	disintegration of the bile ducts epithelium
	15 h	initial decrease in basophilia
	120 h	complete loss of basophilia
Lung	6 h	initial separation of the bronchial and vascular epithelium
	6 h	initial loss of red blood cells
	72 h	general disorganization of pulmonary structures
	150 h	persistence of basophilia

(*) death-delivery time, developed on animal model (rabbits)

Table 2. *Schematic summary of the experimental animal model on post-mortem histological changes in four organs by Shanklin et al. 1964.*

Although Shanklin's means, arguments and criteria were quite rudimentary (just think of the poor reliability of the fetal heartbeat auscultation, as was pointed out, however, by Shanklin itself), the experimental design of this study was well conceived and the conclusion was that the fetal histology could be a potentially good instrument in determining the time of death in the fetus died and retained in utero.

A few years later, in 1970, Babala estimated 6 stages of histological changes of 15 organs in a 110 dead fetuses population, unfortunately without specifying criteria in detail. Babala noticed an acceleration of microscopic changes in cases of fetal hydrops and macrosomia, while he recorded a slowdown in cases of prematurity and anencephaly. As Shanklin, Babala concluded that histology had proved to be an excellent tool for the diagnosis of the time of fetal death.

Momentarily set aside the experiments of Shanklin and Babala, in 1971 Langley published a work on perinatal necropsy methodology. Speaking about external examination, he pointed out that the start of skin breakdown could be observed approximately 8 hours after death; then subdivided the phenomenon evolution in degrees (I, II and III) of maceration. The same three-stage thanatological evaluation system (known as "Langley Criteria") was corrected by Bain in 1974 because, according to his observations, at least 48 hours of intrauterine retention after death were required to observe extensive peeling of the skin (Table 3).

A year later, in 1975, Craig and Potter dealt with the issue of fetal intrauterine post-mortem maceration with a purely descriptive approach, without providing any chronological indications, if not a single observation in addition to what has already

been stated previously from Langley and Bain: after a intrauterine retention of at least 7-10 days the color of the fetus began to change from deep red to brownish-olive.

Maceration degree		**Macerative macroscopic findings**	**Death-delivery time**	
Langley	**Bain**		**Langley**	**Bain**
0	*slight*	reddened skin with "parboiled" appearance	< 8 hours	0-48 hours
I	*slight*	peeling of the skin	> 8 hours	0-48 hours
II	*moderate*	extensive peeling of the skin, reddish flows in pleural and peritoneal serous	*later still*	2-7 days
III	*prolonged*	brownish-yellow liver, turbid flows, mummification	*prolonged*	> 8 days

Tablela 3. *Chronological correlation between the degree of fetal maceration and death-delivery interval (according to Langley 1971 and Bain 1974).*

From the outset it was clear, however, that the limit of these pioneering observations was represented by their empiricism, since they were not based on instrument and objective assessment but purely on author's personal experience. These reasons led Becker et al., in 1989, to say that "*a maceration graduation system is of limited value because it does not provide a precise correlation between the range of intrauterine death and birth*" (from Umani Ronchi et al. 2002). Despite the obvious limitations, we must recognize to Langley work a schematic, mnemonic and particularly intuitive structure, and even today there is often talk of grade I, II and III of skin breakdown referring to Langley Criteria, although this terminology is now used more with descriptive purposes rather than chronological.

The following years were characterized by a stop in the issue on fetal thanatology. In his 1984 textbook of perinatal pathology, Wigglesworth resumes the simple but effective 1922 Strachan description to offer a rather rough chronological

subdivision once again based on his own experience rather than on experimental trials. The author points out that the peeling of the skin may be present from about 6 hours after death, after 24 hours there are bubbles with fluid content between the epidermis and the dermis, after "a few days" hemolysis causes a reddish discoloration of internal organs and there are flows in the serous cavities, and after 5-7 days cranial vault bones have been completely separated. If the fetus remains inside the uterus for more than a week the coloring of the skin and tissue changes from red to yellow-brownish. Finally, Wigglesworth concludes in a somewhat rushed way that "… *it is not possible to time the changes of maceration with any degree of precision. However, it is possible to recognize gross discrepancies between the time at which the fetus was said to have died and the extent of maceration*".

It is also worth mentioning a 1989 study by Hill et al. about the correspondence between the autopsy results and fetal ultrasound images with dubious interpretation. The thanatological interest discussed by the authors is that it is not derived from autopsy findings but from ultrasound images: they observed that epidermis integrity of the fetus is lost within 3-4 days and during the same time a subcutaneous edema of the scalp can be seen; a 5 mm thick edema may be present from the 5th day after death.

A few years later, in 1991, Wigglesworth and Singer edited another textbook of fetal and perinatal pathology, leaving to Singer and Macpherson the chapter about fetal death and the approach to macerated fetus autopsy. With a greater work of literature review, the authors define a thanatological chronological succession of fetal death and associated macerative phenomena different from previous criteria, certainly more complete and extensive but unfortunately still strongly influenced by their personal experience. The "parboiled" appearance of the skin (the famous Langley grade 0) is

observed as early as 4 hours after death. The well-known successive changes (initial flaking, fluid-filled blistering between the epidermis and dermis, brownish-red tissue discoloration, joint laxity) are not chronologically stratified. The next step is already 4-5 days after death, when overlapping of the cranial bones is observed. After a week of intrauterine retention the intestinal wall can dissolve by autolysis and release meconium into the abdominal cavity and the skin color becomes brownish-olive. These changes are generally accelerated in fetuses with cystic hygromas, hydrops and all the conditions associated with tissue edema. The authors also try to summarize a possible chronology of autolytic post-mortem histological changes: liver, intestine and pancreas are in autolysis within 1-3 days with loss of histological details, kidneys are more resistant and the autolysis starts after 48 hours. Even in this case the conclusion is once again straightforward: "*The duration of carriage* in utero *after fetal death is extremely difficult to establish by either macroscopic or microscopic examinations. Only crude estimates are possible*".

The first authors (and the only so far) to analyzed in an experimental and extended way the problem of estimating the time of intrauterine death on the basis of macroscopic and microscopic examinations scientifically valid were Genest et al., who published in 1992 three retrospective studies on cases of fetal death accurately ascertained by serial ultrasound or Doppler examinations. In the first contribution they reviewed histological preparations of fetal organs (150 cases), in the second study they examined histological specimens of the placenta (71 cases) and in the third they have reassessed the external examination of the fetus basing on the available pictures (86 cases). On the basis of overall observations the authors divided the predictive chronological criteria of the interval between fetal death and birth as "good",

"intermediate" and "poor". Genest et al. based their contributions on previous stillbirth studies which, however, were still an individual authors "suggestions": the real capacity of these changes in accurately identify the time of fetal death had never been systematically evaluated with a strong experimental protocol. The methodology used was the same in the three studies: the authors randomly divided available cases into two subgroups: learning cases and test cases. They first examined the group of learning cases and from these observations they derived the criteria (good, intermediate and non-significant); then they applied these criteria to the study of test cases, leading to a further redefinition of the their validity.

For their importance, it is useful to exhibit in an analytical way the results of the articles by Genest et al. about histology of organs and external fetal examination, postponing the analysis of the histological study of the placenta in a later section of this chapter.

With regard to the histological examination of the fetal organs (Genest et al. 1992 I), the authors collected 150 cases in which the condition of the fetus (last relief of vitality, first finding of death) was known and confirmed with ultrasound or Doppler; cases with an interval longer than 7 days between the birth and the autopsy were excluded. These cases were then randomly divided into 100 learning cases and 50 test cases. Starting with learning cases, histological sections of all fetal organs were stained with hematoxylin-eosin and then collectively evaluated by all the authors in chronological order from the lower to the higher birth-death time, in order to develop a sequential autolytic pattern for each organ: 45 histological criteria for 15 organs were established and can be enclosed into five general groups:

1. loss of nuclear basophilia in at least 1% of the cells;
2. maximum loss of nuclear basophilia (100% of the cells);
3. loss of cartilage matrix basophilia in the tracheal and bronchial cartilage;
4. nuclear karyorrhexis in cortical thymic lymphocytes;
5. epithelium exfoliation of the mucous membrane of the bronchi, gastrointestinal tract and uterus.

The authors then calculated the validity and effectiveness of each histological variable (sensitivity, specificity, positive predictive value) as a possible diagnostic test for the following retention times (death-birth time): less than 2 hours, at least 2 hours, at least 4 hours, at least 6 hours, at least 8 hours, at least 12 hours, at least 18 hours, at least 24 hours, at least 36 hours, 48 hours, at least 72 hours, at least 96 hours, at least 1 week, at least 2 weeks, at least 3 weeks, at least 4 weeks and at least 8 weeks: between 45 histological criteria considered, 23 showed a good correlation with intrauterine retention time in terms of sensitivity, specificity and positive predictive value (Table 4).

To determine how fetal histology could be affected by factors other than death-birth time, all histological criteria were also evaluated for a possible association with the following characteristics and circumstances: sex of the fetus, fetal infection, hypoxia/severe stress, hydrops, twin pregnancy, interval between expulsion and autopsy exceeding 24 hours, gestational age at death (less than 25 weeks, 25-35 weeks, more than 35 weeks). Among these, sex , infection, twin pregnancies, and hypoxia did not affect the histological criteria. Histological changes in the fetuses appeared to slow down in very preterm (less than 25 weeks) and accelerated in fetuses with hydrops, advanced gestational age (greater than 35 weeks) and time interval between birth and

autopsy more than 24 hours. The authors, however, specify that these data were derived by examining a few cases and that therefore the real influence of these factors on fetal histology was still unclear.

Histologic criteria *(loss of nuclear basophilia, except *)*	**Death-birth interval**	**Sensibility**	**Specificity**	**Positive predictive value**
Kidney: cortical tubules (a)	≥ 4 h	0.983	0,800	0.934
GI apparatus: mucosal epithelium (a)	≥ 8 h	0.966	0.875	0.952
* Lung: bronchial mucosa epithelial exfoliation	≥ 18 h	1.000	0.903	0.946
Lung: bronchial cartilage matrix (a)	≥ 24 h	0.820	0.919	0.932
Liver: hepatocytes (a)	≥ 24 h	0.957	0.902	0.957
* Thyme: cortical lymphocytes karyorrhexis	≥ 24 h	1.000	0.850	0.857
Heart: ½ internal myocardium (a)	≥ 24 h	0.909	0.919	0.930
Adrenal gland: fetal cortex (a)	≥ 24 h	0.762	0.897	0.889
Pancreas (b)	≥ 36 h	0.917	0.900	0.753
Thyme: lymphocytes (a)	≥ 48 h	0.958	0.837	0.742
Kidney: glomeruli (a)	≥ 48 h	0.806	0.942	0.893
Heart: ½ external myocardium (a)	≥ 48 h	0.839	0.915	0.867
GI apparatus: entire wall (a)	≥ 72 h	0.966	0.960	0.933
Adrenal gland: adult cortex (a)	≥ 72 h	1.000	0.946	0.864
Lung: bronchial epithelium (a)	≥ 96 h	0.920	0.945	0.885
Liver (b)	≥ 96 h	0.917	0.929	0.846
GI apparatus (b)	≥ 1 week	0.950	0.983	0.950
Adrenal gland (b)	≥ 1 week	0.929	0.917	0.722
Trachea: chondrocytes (a)	≥ 1 week	0.750	0.963	0.750
Lung: alveolar wall (a)	≥ 2 week	0.938	0.958	0.833
Kidney (b)	≥ 4 week	1.000	0.976	0.750
Lung (b)	≥ 8 week	0.900	0.988	0.900
Brain: cortical neurons (a)	≥ 8 week	0.714	1.000	1.000

(a) initial loss of nuclear basophilia (1% cells)
(b) maximum loss of nuclear basophilia (100% cells)

Table 4. *Histological findings related to the determination of the time of fetal death in 100 learning cases (Genest et al. 1992, I).*

After this phase, the authors used 50 test cases to determine the accuracy of the 23 histological criteria with good correlation developed by 100 learning cases. All sections of the 50 test cases were observed individually in a blind test by one of the authors and evaluated as if they were each a single diagnostic test, regardless of other

histologic findings in the same fetus: 10 of the 23 criteria resulted "good predictors" for the death-birth interval estimation (sensitivity, specificity and positive predictive value of at least 0.875), while among the remaining 13 criteria, 8 have proven to be poor predictors and 5 achieved intermediate values (Table 5).

Histologic criteria *(loss of nuclear basophilia, except *)*	**Death-birth interval**	**Sensibility**	**Specificity**	**Positive predictive value**
Good predictors				
Kidney: cortical tubules (a)	≥ 4 h	0.971	0,889	0.971
Liver: hepatocytes (a)	≥ 24 h	1.000	0.920	0.889
Heart: ½ internal myocardium (a)	≥ 24 h	0.938	1.000	1.000
Heart: ½ external myocardium (a)	≥ 48 h	1.000	0.964	0.909
Lung: bronchial epithelium (a)	≥ 96 h	1.000	0.973	0.909
Liver (b)	≥ 96 h	0.909	1.000	1.000
GI apparatus (b)	≥ 1 week	0.900	1.000	1.000
Adrenal gland (b)	≥ 1 week	1.000	1.000	1.000
Trachea: chondrocytes (a)	≥ 1 week	0.889	1.000	1.000
Kidney (b)	≥ 4 week	1.000	0.976	0.875
Intermediate predictors				
GI apparatus: mucosal epithelium (a)	≥ 8 h	0.930	0.800	0.900
Adrenal gland: fetal cortex (a)	≥ 24 h	0.813	0.957	0.929
Pancreas (b)	≥ 36 h	0.714	1.000	1.000
GI apparatus: entire wall (a)	≥ 72 h	1.000	0.909	0.769
Lung: alveolar wall (a)	≥ 2 week	1.000	0.949	0.800
Poor predictors				
* Lung: bronchial mucosa epithelial exfoliation	≥ 18 h	1.000	0.588	0.720
Lung: bronchial cartilage matrix (a)	≥ 24 h	0.941	0.625	0.640
* Thyme: cortical lymphocytes karyorrhexis	≥ 24 h	1.000	0.429	0.429
Thyme: lymphocytes (a)	≥ 48 h	1.000	0.880	0.571
Kidney: glomeruli (a)	≥ 48 h	1.000	0.821	0.688
Adrenal gland: adult cortex (a)	≥ 72 h	1.000	0.875	0.714
Lung (b)	≥ 8 week	1.000	0.976	0.875
Brain: cortical neurons (a)	≥ 8 week	0.750	0.970	0.750

(a) initial loss of nuclear basophilia (1% cells)
(b) maximum loss of nuclear basophilia (100% cells)

Table 5. *Histological findings in the test cases group: validity as specific diagnostic criteria for defining death-birth interval (Genest et al. 1992, I).*

After the histological findings were analyzed as independent criteria, they were subsequently tested as criteria in association with each other on the 50 test cases. When all 23 criteria were used, 54% of the cases were correctly placed in one of the windows of fetal death interval. When the 8 poor predictors were excluded, the number of cases correctly classified rose to 64%. Finally, by limiting the analysis to the 10 best predictors the success rate increased to 86%.

To conclude, the most important histological criteria common to the 10 best predictors of the time of death was found to be the more or less extensive loss of nuclear basophilia (i.e. loss of the nucleus) in histological preparations stained with standard hematoxylin-eosin staining (as indeed had already been highlighted by Strachan in 1922). The advantages of the histological examination of fetal organs are represented by the fact that it is based on objective data of simple detection, such as the loss of nuclear basophilia and its quantification percentage. Furthermore, the method can also be applied to archives material. The main limitations are traced to the need to perform a precise differential diagnosis with other causes of cellular necrosis and nuclear loss, such as ischemia-anoxia, infection, etc.

With regard to the external examination of the fetus (Genest and Singer 1992, III), the authors reviewed photographs from 86 stillbirths with time of death assessed by ultrasound or Doppler examination, in order to determine, as in previous work, good predictive criteria for the determination of the time of fetal death. The experimental method adopted by the authors is similar to the above: 86 cases were randomly divided into 60 learning cases and 26 test cases. The authors initially examined the photographs of the 60 learning cases in chronological order (from lower

to higher birth-death time) to define a set of findings correlated with the time of diagnosis of fetal death, then statistically evaluated to define sensitivity, specificity and positive predictive value (Table 6). Only the predictors with statistical values greater than 0.800 were then used for subsequent analysis of the 26 test cases.

Macroscopic criteria	Death-birth interval	Sensibility	Specificity	Positive predictive value
Good predictors				
Desquamation ≥ 1 cm	≥ 6 h	0.853	0.812	0.921
Brown or reddish cord discoloration	≥ 6 h	0.947	0.867	0.947
Face, back or abdomen desquamation	≥ 12 h	0.864	0.905	0.941
Desquamation ≥ 5% of the body	≥ 18 h	0.862	0.920	0.926
Desquamation of 2 or more of 11 zones (*)	≥ 18 h	0.931	0.920	0.931
Brown or reddish skin discoloration	≥ 24 h	0.828	0.928	0.923
Moderate or extended desquamation	≥ 24 h	0.896	0.857	0.867
Mummification	≥ 2 week	0.888	1.000	1.000
Intermediate predictors				
Any desquamation	≥ 3 h	0.878	0.667	0.923
Reddish skin discoloration	≥ 4 week	1.000	0.962	0.714
Poor predictors				
Cranial bones compression	≥ 36 h	0.619	0.935	0.866
Desquamation ≥ 10% of the body	≥ 48 h	0.904	0.857	0.791
Desquamation ≥ 75% of the body	≥ 72 h	0.529	0.945	0.818
Wide open mouth	≥ 1 week	0.700	0.837	0.500

(*) skull, face, neck, chest, abdomen, back, upper arms, hands, legs, feet, scrotum

Table 6. *Validity of macroscopic findings as indicators of the intrauterine fetal retention period in 60 learning cases (Genest and Singer 1992, III).*

The 8 best predictors derived from the 60 learning cases were also analyzed for possible associations with factors external to the period of intrauterine retention (gestational age at death less than or greater than 28 weeks, fetal hydrops, infection, time interval from death to autopsy great than 24 hours, signs of fetal acute/chronic distress): only the presence of fetal hydrops was found to speed up the macerative process. Subsequently, the good predictors of learning cases were tested on 26 test

cases, considering each individual as an indicator, and subjected to statistical analysis (Table 7).

Macroscopic criteria	Death-birth interval	Sensibility	Specificity	Positive predictive value
Good predictors				
Desquamation ≥ 1 cm	≥ 6 h	0.857	1.000	1.000
Face, back or abdomen desquamation	≥ 12 h	0.800	1.000	1.000
Desquamation ≥ 5% of the body	≥ 18 h	0.800	1.000	1.000
Desquamation of 2 or more of 11 zones (*)	≥ 18 h	0.900	0.923	0.900
Mummification	≥ 2 week	1.000	1.000	1.000
Intermediate/poor predictors				
Brown or reddish cord discoloration	≥ 6 h	0.833	0.667	0.768
Brown or reddish skin discoloration	≥ 24 h	0.800	0.800	0.727
Moderate or extended desquamation	≥ 24 h	0.700	0.933	0.875

(*) skull, face, neck, chest, abdomen, back, upper arms, hands, legs, feet, scrotum

Table 7. *Validity of macroscopic findings as indicators of the intrauterine fetal retention period in the 26 test cases (Genest and Singer 1992, III).*

The 8 criteria defined as relatively good predictors of 60 learning cases have been reduced to 5 once passed to the application of test cases. As can be seen from Table 7, the majority of these predictive criteria regards the degree of skin desquamation or maceration. This figure is usually easily detectable by the pathologist, also once carried out the post mortem examination from the description or photographic surveys of the fetus. Genest and Singer also note, finally, that the predictors encumbered by a greater number of incorrect results are those related to the skin or cord color, because the subjective evaluation of color, especially passing from reddish to brownish, has resulted the less reproducible item between different observers.

For the first time, therefore, the chronological sequence of changes in fetal external maceration was validated with experimental observations, without perpetuating the repetition of empirical data derived from the experience of the individual author or the conclusions of other authors. An overall comparison of all the "good predictors" of the three studies by Genest et al. is outlined in Table 9.

In 1995 Genest himself edited a very extensive chapter about the macerated fetus in the manual by Reed et al., completing a remarkable work of synthesis of all the authors who have dealt with the issue and with his personal experiences above. Intentionally proceeding with a maximum simplification and schematization, the evolution of the macerative process of the fetus in utero could be summed up in three main steps: within 24 hours, within 1 week, and more than 1 week.

- Recent maceration (less than 24 hours of uterine retention): sagging skin with sliding of epidermis on the dermis, expansion of the skin on the support surface to the fetus lying (start at 6 hours after death), "parboiled" appearance of the skin (early manifestations at 4-6 hours after death).
- Intermediate maceration (uterine retention from 1 to 7 days): skin bubbles with progressive rupture and dermal exposure (early events at 24 hours after death), red-brown color of the viscera and small hemolysis flows in the cavity (increase from 24 hours to 5 days), overlapping of the cranial bones for separation of bone from dura mater, joint laxity, semi-liquid consistency of the brain (full manifestation after 5 days of death).

- Advanced maceration (uterine retention more than a week): transformation of skin color from red to brown (early events from 7-10 days after death), olive coloration of the skin (a few weeks after the event), gray color and "mummified" appearance of the skin (advanced event, more than 2 weeks).

The same chronological sequence, with minor variations, was later reproduced by Moore in 2001 and Fulcheri et al. in 2006, reflecting the fact that in the first half of the '90s anything significant was added to what was already concluded by Genest.

Even in the medico-legal field, meanwhile, manuals and texts continue to bring back old descriptions and assessment chronological criteria. Among these stands out the 1997 work by Gerin et al., which publishes a chronological sequence that is significant in the effort to set up a personal rough guide, although again based only on the personal experiences of the authors:

- until the 3rd day the appearance is almost normal, the skin is intact but has a cupric color initially confined to the face and neck, with signs of blood infiltration to the base of the umbilical cord, as well as a decrease consistency of the brain;
- the 6th day the fetus takes on a "sagging" skin appearance, intensely soaked, presents diffuse cupric staining, characteristic blood-serum flows are produced in serous cavities, the brain is extremely soft, the viscera have extremely low consistency;

- the 12th day the body is extremely flaccid, the skin takes on a widespread blackish color, presenting extensive areas of epidermal detachment, the viscera show partial colliquation, the brain has very low consistency;
- beyond the 20th day the autolytic processes cause colliquative disintegration of the soft parts.

The latest study found in the literature about fetus maceration dates back to 2005 by Maroun and Graem. The aim of the authors was to provide autopsy standard of body parameters and organs weights in macerated and not macerated fetuses. The division of the macerative process for the distribution of the population turned out to be very approximate, based on purely observational surveys: the three groups were called "mild", "moderate" and "marked" ("*Mild maceration was defined as bullae or skin slipping on extremities or small parts of the face or trunk. Moderate maceration was defined as extensive skin slipping and reddish discoloration of the skin and umbilicus. Marked maceration was defined as brownish, tan, or yellowish skin discoloration, overlapping cranial bones, loose joints, and/or mummification*"). The authors, however, to prove that these signs of maceration increased with the progression of uterine retention time, subjected the populations of macerated fetuses (3 groups) and not macerated fetuses to a statistical correlation between the variables: not only they were able to positively correlate the advancement of the signs of maceration with the progress of the uterine retention time, but they also succeeded in statistically approximating the uterine retention time for each group: a few days for the "mild" group, about 2 weeks for the "moderate" group and over 4 weeks for the "marked" group. This estimation shifts several days ahead the macerative process as discussed by other authors. To get a more accurate result, probably the

groups into which the macerated fetuses were divided could be better characterized and expanded: it would certainly be of great interest to repeat the statistical analysis with more accurately identified groups.

THE PLACENTA

The post-mortem study of the placenta and its sampling for histology are essential moments in a complete and comprehensive post-mortem investigation of the fetus and for the diagnosis of fetal death (for a review see: Marchetti et al. 2007).

Placenta is not affected by macerative phenomena after fetal death, as it continues to receive nourishment from the maternal circulation. But even if it retains the characteristics of a vital organ, the placenta is affected by important morphological changes after fetal death, primarily represented by atrophic and fibrotic phenomena (Dimmick and Kalousek 1992; Fox 1968; Genest 1995; Moore 2001; Singer and Macpherson 1991; Wigglesworth 1984). These changes, especially when massively affecting the placenta, can be misinterpreted as already present at the time of fetal death or as etiologic phenomena directly related to its occurrence (Fox 1968).

Although one of the first scientific papers focused specifically on changes in retained placenta in the uterus after the death of the fetus dates back to 1896 by Eden (Thomson 1927), this phenomenon has long been neglected and only from the '60s can be found scientific contributions. Wilkin in 1965 revealed a marked increase in the number of syncytial knots and collagen in the villi after the death of the fetus, together with a fibrin intervillous deposition and a proliferation of endothelial cells in fetal arteries. The following year Emmrich studied a group of placentas of macerated

fetuses: his observations demonstrated the presence of multiple thrombosis in the fetal arteries, marked calcifications, leukocyte infiltration and fibrosis of the villi (from Fox 1968). Davies and Glasser in 1967 observed by electron microscopy the placenta of a dead fetus retained in utero for two weeks; the main findings were: thickening and sclerosis of the fetal arterial walls, villous fibrosis, thickening of the basal membrane of the trophoblast and marked increase of Langhans cells in the villi.

The first important modern contribution to the subject of placental post - mortem changes is traced back to the 1968 work by Fox: the author, starting from the evidence that some placental histological changes were related to the cessation of fetal movement and were to be distinguished from those which may be present before the death of the fetus, undertaken a study with the declared intention not only to identify these changes, but also to approximate the amount of time of uterine retention they takes to occur. The author studied the placentas of 36 fetuses of at least 37 weeks of gestation died of anoxia, in which the retention time of the uterus before the expulsion was known with precision (21 cases within 24 hours from death, 9 cases between 1 and 7 days, 6 cases more than one week). For each placenta about 400 villi were histologically examined for the following characteristics: syncytial knots, Langhans' cells, stromal fibrosis, Hofbauer cells, stromal edema, thickening of the basal membrane of the trophoblast. Comparing the results obtained with the physiological changes of normal placentas, these changes were stratified as "normal", "high" or "very high". Similarly, Fox taken into account the presence of fetal arterial thrombosis, obliterative endarteritis or fibromuscular sclerosis. These alterations were classified as "mild" or "severe". The microscopic features which best correlated with the time of

fetal death proved to be a marked hyperplasia of fibrous and muscle layers of the vessel wall and the presence of subintimal fibrotic tissue projecting into the lumen. More specifically, Fox did not notice the presence of fibromuscular sclerosis in 21 placentas of fetuses died within 24 hours. However, he observed different degrees of luminal stenosis in those deaths occurred by more than 24 hours. Other characteristics examined (thickening of the basal membrane of the trophoblast, increased number of syncytial knots, fibrosis and stromal edema) were present in greater number, though not significantly, in the placentas of fetuses held in utero for more than a week.

In subsequent years, little was added to these considerations. In his 1984 textbook, Wigglesworth again underlines the importance of the trophoblast changes (increased number of syncytial knots, villous cell proliferation, basal membrane thickening), villous stromal fibrosis and progressive fibromuscular sclerosis of the fetal arteries such as characteristic elements of the fetal circulation cessation in the villi, pointing out that such changes occur rapidly and reach an advanced stage after about 5 days of intrauterine retention.

In 1991, Singer and Macpherson added to the considerations by Fox and Wigglesworth the observation that the villi collagen, intervillar fibrin, proliferation of fetal endothelial cells, calcium deposits and edema of the villi show a slight increase during the first week, and then become significant until the obliteration of the villi capillaries.

Relevant in this review is the experimental model described by Silver et al. in 1988, in which placental cultures were used to simulate the physiological environment of retained placenta in the uterus after the death of the fetus. The chorionic tissue from

15 placentas of fetuses born without disease were maintained in culture for varying periods of time and subsequently histologically analyzed. The majority of cases developed abnormalities in chorionic blood vessels after 3 days in culture: vascular anomaly ("*hemorrhagic endovasculitis-like lesion*") was judged to be present at the occurrence of at least three of the following histological changes: intravascular karyorrhexis, presence of endoluminal septa, myointimal proliferation, intravascular fibrin deposition, transmural extravasation of erythrocytes or leukocytes.

It has already been emphasized the importance of the work done by Genest et al. in 1992, which for the first time are employed in an experimental way the problem of the time of death of the fetus in utero. It remains here to discuss the third study, the one concerning the histological examination of the placenta (Genest 1992, II), whose experimental base remains similar to that of the other works discussed above, to which we refer. The author retrospectively examined the placentas of 71 dead fetuses retained in utero, with time of death assessed by ultrasound or Doppler examination, in order to determine good predictive criteria for determining the age of fetal death. The 71 cases were randomly divided in 51 learning cases and 20 test cases. Initially, the author analyzed the histological sections of 51 learning cases, stained with Hematoxylin-Eosin, to define the presence of the following 15 histological features:

- Umbilical cord
    1. Vasculitis
    2. Loss of nuclear basophilia in the stromal cells
    3. Loss of nuclear basophilia in vascular smooth muscle
- Chorionic plate
    4. Acute chorioamnionitis
    5. "Meconium-laden" macrophages in the corium
- Main villi
    6. Abnormalities of the vascular lumen (multifocal/extended)

- Terminal villi: stroma
    7. Infarcts
    8. Edema of the villi
    9. Extensive fibrosis of the villi
    10. "Pulviscolar" stromal microcalcifications
- Terminal villi: blood vessels
    11. Nucleated red blood cells
    12. Intravascular karyorrhexis
- Terminal villi: trophoblast
    13. Increased syncytial knots
    14. Thickening / calcification of the basal membrane
    15. Increased number of cytotrophoblastic cells

The loss of nuclear basophilia was defined as the absence of blue staining in at least 1% of the cells; vascular abnormalities (occluding or suboccluding fibro-intimal hypertrophy) were defined as "multifocal" if present in 10-25% of the villi, "extended" if present in more than 25% of the villi. This set of findings correlated with the diagnosis of time of fetal death was subsequently statistically assessed to define the sensitivity, specificity and positive predictive value with respect to the following classes of birth-death time: less than 2 hours, at least 2 hours, at least 4 hours, at least 6 hours, at least 8 hours, at least 12 hours, at least 18 hours, at least 24 hours, at least 36 hours, at least 48 hours, at least 1 week, at least 2 weeks, at least 3 weeks, at least 4 weeks and at least 8 weeks. Among the 15 histological criteria considered, 8 showed no correlation with the timing of fetal death ("meconium-laden" macrophages in the corium, vasculitis in the umbilical cord, acute chorioamnionitis, infarcts of the villi, villous edema, nucleated red blood cells, increased syncytial knots, increased cytotrophoblast cells). Of the remaining 7 criteria, only 3 could be considered good predictors, having obtained values of sensitivity, specificity and positive predictive value greater than 0.750 (Table 8).

Histologic criteria	Death-birth interval	Sensibility	Specificity	Positive predictive value
Good predictors				
Intravascular karyorrhexis	≥ 6 h	0.935	1.000	1.000
Vascular luminal anomalies of the main villi				
Multifocal	≥ 48 h	0.944	1.000	1.000
Extensive	≥ 2 week	0.777	0.976	0.875
Extensive fibrosis of the villi	≥ 2 week	1.000	0.928	0.750
Poor predictors				
"Pulviscolar" stromal calcifications	≥ 24 h	0.478	0.969	0.916
Necrosis of the Wharton's jelly	≥ 48 h	0.733	0.843	0.688
Calcification/thickening of the basal membrane of the trophoblast	≥ 48 h	0.333	0.928	0.667
Necrosis of umbilical cord vessels	≥ 1 week	0.600	1.000	1.000

(*) skull, face, neck, chest, abdomen, back, upper arms, hands, legs, feet, scrotum

Table 8. *Validity of seven histological findings as indicators of the intrauterine fetal retention period in the 51 learning cases (Genest 1992, II).*

Genest also pointed out that as regards the intravascular karyorrhexis, leukocytes were the most represented cell population. The 3 best predictors derived from the 51 learning cases were subsequently tested on the 20 test cases, considering each individual as an indicator, confirming their value in determining the time interval between fetal death and birth. Finally, the author also analyzed the placentas of 4 term infants without pathologies stored at 5°C for varying periods of time (up to 14 days) in order to identify any artifacts from refrigeration, identifying only the presence of endothelial "free" cells in the lumen of the vessels after 7-14 days of refrigeration. The good predictors, therefore, were not affected. The advantages of histological examination of the placenta are represented by objectivity and easy detection of vascular anomalies and stromal fibrosis, even on archive material. The limits, however, are related to the fact that such alterations are sometimes associated with thrombosis of some vessels of main villi; differential diagnosis, however, is not difficult because in

this case the alterations are only in the district of the thrombosed vessel, while those derived from the death of the fetus are multifocal.

An overall comparison of all the "good predictors" of the three studies by Genest et al. is outlined in Table 9.

Death-birth interval	External fetal examination	Organs histology	Placenta histology
≥ 4 h		Kidneys: loss of nuclear basophilia of cortical tubules (> 1%)	
≥ 6 h	Desquamation ≥ 1 cm		Intravascular karyorrhexis of the villi (exp. leukocytes)
≥ 12 h	Face, back and abdomen desquamation		
≥ 18 h	Desquamation ≥ 5% body Desquamation of 2/+ zones		
≥ 24 h		Liver: loss of nuclear basophilia of hepatocytes (> 1%) Myocardium: loss of nuclear basophilia of the inner half (> 1%)	
≥ 48 h		Myocardium: loss of nuclear basophilia of the outer half (> 1%)	Multifocal abnormalities in vascular lumens of the main villi
≥ 96 h		Bronchi: loss of nuclear basophilia epithelium (> 1%) Liver: maximum loss of nuclear basophilia	
≥ 1 week		GI: maximum loss of nuclear basophilia Adrenal gland: maximum loss of nuclear basophilia Trachea: loss of nuclear basophilia of chondrocytes (> 1%)	
≥ 2 weeks	Mummification		Extensive vascular lumen anomalies of the main villi Extensive fibrosis of the villi
≥ 4 weeks		Kidneys: maximum loss of nuclear basophilia	

Table 9. *Comparison of the "good predictors" criteria of the three studies by Genest et al. and temporal correlation with death-birth interval (Genest et al. 1992, I, II, III).*

A few years later, Genest himself (Genest et al. 1997) alert pathologists to take extreme care not to confuse changes in vascular smooth muscle of the umbilical cord, secondary to uterine retention of dead fetus, with the remains of an ascending bacterial infection of the amniotic fluid. Microscopically, in fact, many degenerative cells of the vessels muscular wall can mimic the irregular shape and staining of neutrophils ("pseudo-vasculitis"). To distinguish these cells from the real neutrophil Genest et al. suggest to evaluate the distribution: very regular in "pseudo-vasculitis", localized in case of true vasculitis.

The most recent contribution to the diagnosis of fetal death based on the histological study of the placenta is by Jacques et al., published in 2003. The authors retrospectively evaluated 36 placentas from therapeutic abortions, in which the death-birth time was known, well documented and relatively short, to identify early changes after the death of the fetus. They histologically analyzed a set of placental modifications (similar, but not identical, to those of Genest), subsequently subjected to statistical analysis for correlation with known retention time: less than 12 hours, 12-24 hours, 24-36 hours and more than 36 hours. The study identified the statistical validity of three variables: intravascular karyorrhexis, especially by leukocytes, already observed after 6 hours; degeneration of vessels muscle fibers of the umbilical cord, completely absent before 12 hours; vascular intraluminal anomalies in the main villi (for which positivity a value exceeding 5% was required, where the study by Genest required at least 10-25%), observable after 36 hours. According to Genest, microcalcifications and stromal thickening or calcification of the trophoblast basal membrane were not found significant in correlation to the time of fetal death.

It is worth mentioning for completeness two studies that dealt with the kinetics of meconium phagocytosis by placental macrophages, because this finding was present among those used by Genest in 1992, although not proved to be correlated to the uterine retention time in fetal death. Indeed, as we will see, this information belongs more to the field of the timing of fetal distress than to thanatology.

The first important study was undertaken in 1985 by Miller et al., who created placental and umbilical cord in-vitro cultures dirtied by meconium solutions at different concentrations (5%, 10%, 20%). Considering closer to reality the cultures incubated with 10% of meconium, the authors observed contamination of the umbilical cord after 15 minutes of exposure, and the contamination of the placenta was observed only after an hour. The depth of penetration of meconium in placental membranes and the consequent uptake by macrophages resulted independent of the concentration of meconium, being, however, related to the exposure time: meconium-stained macrophages in the amniotic fluid were observed after one hour of exposure, and in chorionic membranes after 3 hours; no macrophage was found in the umbilical cord (Table 10).

In 2009 Funai et al. returned on the topic with the specific intent to "correct" the time estimates of Miller et al., considered too short. The authors used a different experimental in-vitro model considered more adherent to reality (for example, a 10% solution of meconium was not used, but pure meconium added directly to the culture). The study confirmed that the depth of penetration of meconium in placental membranes is directly correlated only with the duration of exposure and not with the concentration of meconium, but no macrophage is observed in the amniotic fluid

before 24-48 hours of exposure. The results of the study and a comparison with the work by Miller et al. are summarized in Table 10.

	Miller et al. 1985	Funai et al. 2009
≥ 15 min.	Contamination of the umbilical cord	
≥ 1 h	Contamination of the placenta; macrophages with meconium in the amniotic fluid; pseudostratification of the amniotic columnar epithelium	
≥ 3 h	Macrophages with meconium in the corium; disorganization of amniotic epithelium	Free meconium in amniotic epithelium and in connective tissue
≥ 12 h		Early uptake of meconium by macrophages
≥ 24 h		Greenish discoloration of the placenta; increase of macrophages and the amount of uptake
≥ 48 h		Brownish discoloration of the placenta; free meconium in the chorion/ decidua

Table 10. *In-vitro experiments for the macrophage meconium uptake kinetics in the event of fetal distress in utero (Miller et al. 1985; Funai et al. 2009).*

In conclusion, as already pointed out, the placenta not only has a clinical and pathological priceless value, but also in the medico-legal disputes often becomes impossible to reach a judgment about the cause of death without a complete analysis of the fetus-placental unity (Jacques et al. 2003; Marchetti et al. 2007, 2008, 2009). However, despite the usefulness of the placenta unanimously accepted also in the diagnosis of the time of fetal death, it is useful to place a call for prudence in judgment, especially in the field of judicial for suspected obstetrical and gynecological responsibility, or in the case of discovery of the placenta alone, already detached from

fetal structures: it is always necessary, in fact, proceed with a comprehensive analysis of all the available information (medical records, analysis of the mother and fetus) being now impossible to express an opinion concerning the history of fetal death only by the analysis of the placenta (Marchetti et al. 2007).

THE MACERATION OF THE CORPSE IN WATER

At the conclusion of this chapter, it is worth dwelling on the medico-legal approach to corpses immersed in liquid medium. While using the same term "maceration", the post-mortal transformations affecting the corpse in the liquid medium are not, of course, neither sterile nor take place at a constant temperature of 37°C. However, there are many points of contact between the fetus maceration and that of the corpse in liquid medium and it would be a mistake to overlook the studies in this area.

In general, the macerative phenomena that are observed in corpses staying in a liquid medium prevail over putrefaction as lower the temperature of the immersion liquid is. In case of permanence for long duration in a liquid environment, from maceration you can gradually move towards saponification (Gerin et al. 1997; Saukko and Knight 2004; Umani Ronchi et al. 2002). Chronological references, as vague, are available in the literature only with reference to the time of immersion in a liquid environment, which does not necessarily coincide with that of death. The epidermis, especially of the hands and feet, already after a few hours of storage in water becomes wrinkled, soft and whitish, until, after several days of immersion, with the possible contribution of mechanical factors of any nature, tends to detach characteristically in

"glove" or "sock" shape, also including the nails and hair. Putrefaction accelerates the detachment of the epidermis from the dermis (Saukko and Knight 2004; Umani Ronchi et al. 2002).

From the literature on the history of maceration significantly different opinions emerge, sometimes sharply contrasting with each other: the influence of environmental factors or intrinsic to the body is considerable and not always well known.

Already in 1895, the professor of forensic medicine Antonio Raffaele, in his textbook "Guida pratica alle perizie medico-legali", discussing the questions related to judicial necropsy of a body pulled from the water , also analyzed the "*judgment on the time that the corpse remained in the water*". Raffaele immediately warns the reader: "... *The examiner must be very reserved in judging the time of death of the notes of putrefactive corpse removed from the water, being many circumstances that can lead to variations on those notes (age, sex, constitution, clothes, seasons), and the judgment must therefore always be approximate*". The author then continues with a series of chronological indications that still impress with their completeness: "*cadaveric rigidity and early bleaching on hands especially in thenar, hypothenar regions, the inner sides of the fingers, from 3 to 5 days. - Progressive softening of the meat for water imbibition, preserving the natural color, from 4 to 8 days. - Swollen face with slight cerulean tinge, soles of the feet epidermis softened milky white, 8 to 12 days. - Very pronounced wrinkles of the skin of the hands, swollen face, mottled red: greenish tint of the upper parts (head, neck, ears), especially towards the upper regions of the back, after 15 days...*", to the description of the saponification almost complete after 4 and a half months, and finally: "*from five months then it is not possible to indicate what is the succession of the phenomena of putrefaction underwater, because the*

alterations stop during saponification, or the body, going down in the bottom of the river, sea or lake, goes towards a complete skeletonization".

For a more recent example, it is shown the chronological indications provided by Gerin et al. in 1997:

- during the first 24 hours is observed bleaching and slight wrinkling of the skin of the fingers, especially the fingertips;
- the phenomenon is accentuated until the 3rd day with involvement of the margins of the fingers, palms and soles of the feet (partial epidermal detachment can start from day 4);
- then the detachment of epidermal "gloves" and "socks" occurs, usually completed between the 7th and the 15th day; the remaining epidermal areas are easily detachable by simply rubbing the skin;
- then there are no reference parameters, for which the estimation of the time of immersion becomes highly approximate.

Other observational studies, reported by Di Maio and Di Maio in 2001, indicate that placing the hands of a dead body in the water at a temperature of 10-18°C, the wrinkling of the fingertips starts after about 20-30 minutes and involves entirely fingers after 50-60 minutes.

What deserves to be deepened here about the maceration of the body immersed in a liquid medium are skin histology studies (all quite old) trying to improve the estimates of the chronological phenomenon: with all the prudence and care that the comparison imposes, these considerations can also offer interesting insights for the histological approach to the fetus maceration.

The first studies on skin histology for dating the period of immersion date back to the '30s, with the works by Ökrös (1938) and Dierkes (1938), although many authors proved immediately pessimistic about the validity of these histological criteria. According to the studies of Ökrös, a reduction of the elastic fibers in the skin and abdominal lumbar indicates a residence time in water of 3-10 weeks. Dierkes said that maintaining a good stainability of elastic fibers in the papillary layer of the foot sole indicates a permanence in water not more than one week (Janssen 1984; Saukko and Knight 2004).

Systematic histological studies on the skin changes associated with the permanence in water taking into account the water temperature, the type of water (fresh/salt), the movements of the liquid medium (sea/river), the age and the thickness of the skin of the subject, were undertaken in 1951 by Schleyer, but did not reveal a linear chronological connection between the time in water and histological changes detectable in the epidermal structures. The only conclusions were that the wrinkling of the epidermis is explained by a general contraction of the elastic fibers system in the superficial corneal layers: the fact that the wrinkling is formed only on the parts of the body with horny layers thick indicates that the "hardness" of the stratum corneum is an important factor in the development of the phenomenon (from Janssen 1984).

In 1980 Motta and Tavani presented at the XXVII National Congress of Legal Medicine the results of an experimental study on the histological and histochemical skin maceration. The authors sampled skin in the plantar region, free of alterations, and subjected it to experimental maceration in fresh water, so that the sample comes in contact with the liquid only through the epidermal surface, for times ranging between

10 and 100 hours. All samplings underwent the following stains: Hematoxylin-Eosin, Van Gieson, Weigert (for elastic fibers), Gomori (for reticular fibers), Garvin et al. (for nucleic acids); on cryostat sections of each sample proceeded to the demonstration of DPN-diaphorase with the method of Naclas et al. The results of the various observations are so schematized:

- periods of immersion of 10-20 hours did not result in appreciable histological or histochemical changes;
- at 30 hours early detachments of the stratum corneum were observed;
- in later times these alterations were gradually more pronounced until the total disappearance of the stratum corneum for times up to 90 hours;
- from 40 hour appeared cytoplasmic and nuclear alterations of the spinous layer, with formation of optically empty perinuclear halos, which have gone larger and larger with the progression of the immersion;
- the DPN-diaphorasic activity up to 30 hours of immersion is present only at a vascular and glandular level, it is markedly decreased from the 40 hours on, to disappear totally over 50 hours;
- for times of 90-100 hours is partially preserved only the granular layer; very late alterations of elastic fibers and reticular have seen in the form of swelling, fragmentation and understainability;
- the Garvin method showed a slow and gradual extinction of ribonucleic acid, beginning with the most superficial dermal layers, structurally less rich, and after about 72 hours the reactions were markedly attenuated at

the surface, with disappearance of RNA in the spinous layer for most of its thickness.

With regard to histology, the only other available observations are those made by Janssen in 1984, who points out the following:

- a good stainability of the elastic fibers of the soles feet skin papillary layer indicates a permanence in water not more than one week;
- a moderate stainability of elastic fibers together with a clear skin wrinkling, in the absence of epidermal detachment, indicate a permanence in water for 2-3 weeks;
- poor or no stainability indicates a duration of immersion more than 4 weeks;
- the preservation of stainability and structure of elastic fibers in lung indicates an immersion period not exceeding two months.

Unfortunately, as with many studies on the chronology of fetus maceration, these observations mostly represent Janssen's empirical considerations. The author concludes, in fact, that "*A histological investigation of the structure and stainability of the elastic fibers can, on the whole, only have a supportive role in the determination of the time a corpse spent in water*".

FETAL CHRONOLOGICAL CRITERIA

	Langley 1971	Bain 1974	Wigglesworth 1984	Singer and Macpherson 1991	Genest et al. 1992, I	Genest et al. 1992, III	Maroun and Graem 2005
≥ 4 h				Parboiled appearance of skin and early peeling of the skin	Kidney: loss of nuclear basophilia of cortical tubules (>1%)		
≥ 6 h			Early skin desquamation			Desquamation ≥ 1 cm	
≤ 8 h	Parboiled appearance of skin						
≥ 8 h	Early skin desquamation						
≥ 12 h						Face, back and abdomen desquamation	
≥ 18 h						Desquamation ≥ 5% body Desquamation in 2/+ zones	
≥ 24 h			Bubbles with liquid content between epidermis and dermis	Liver, intestines and pancreas autolysis with loss of histological detail	Liver: loss of nuclear basophilia of hepatocytes (>1%) Myocardium: loss of nuclear basophilia of the inner half (>1%)		
≤ 48 h		Parboiled appearance of skin and early peeling of the skin					
≥ 48 h				Kidneys autolysis with loss of histological detail	Myocardium: loss of nuclear basophilia of the outer half (>1%)		
Few days			Reddish discoloration of internal organs, serous cavities flows				Bubbles and areas of desquamation at the ends or in small areas of the face and trunk

≥ 4 days				Overlapping of the cranial bones	Bronchi: loss of nuclear basophilia in epithelium (>1%) Liver: maximum loss of nuclear basophilia		
≥ 5 days			Complete separation of the cranial vault bones with overlapping				
≤ 7 days (2-7)	*[later still]* Extensive desquamation and reddish flows in serous cavities	Extensive desquamation and reddish flows in serous cavities					
≥ 7 days			Yellow-brown discoloration of fetal tissues	Presence of meconium in the abdominal cavity, olive-brown discoloration of the skin	GI: maximum loss of nuclear basophilia Adrenal gland: maximum loss of nuclear basophilia Trachea: loss of nuclear basophilia in chondrocytes (>1%)		
≥ 8 days	*[prolonged]* Yellow-brownish liver, turbid flows, mummification	Yellow-brownish liver, turbid flows, mummification					
≥ 2 weeks			Mummification			Mummification	Extensive peeling and reddish discoloration of the skin
≥ 4 weeks					Kidneys: maximum loss of nuclear basophilia		Brownish or yellowish discoloration of the skin, overlapping of cranial bones, mummification

PLACENTAL CHRONOLOGICAL CRITERIA

	Fox 1968	Wigglesworth 1984 Singer & Macpherson 91	Silver et al. 1988	Genest 1992, II	Jacques et al. 2003
≥ 6 h				Intravascular karyorrhexis of the villi (exp. leukocytes)	Intravascular karyorrhexis of the villi
≥ 12 h					Degeneration of smooth muscle of umbilical cord (myocytolysis)
≥ 24 h	Hyperplasia of fibrous and muscle tissue of fetal vessel wall; early increase of fibrous tissue in the subintimal vascular lumen				
≥ 36 h					Abnormalities of main villous vascular lumen (> 5%)
≥ 48 h				Multifocal abnormalities in main villous vascular lumens	
≥ 3 days			Vascular abnormalities (intravascular karyorrhexis, intraluminal septa, myointimal proliferation, intravascular fibrin deposition, transmural extravasation of erythrocytes or leukocytes)		
≥ 5 days		Increased number of syncytial knots, villous cell proliferation, thickening and mineralization of the basal membrane, villous stromal fibrosis, fibromuscular sclerosis of fetal arteries			
≥ 7 days	Thickening of the trophoblast basal membrane; increased number of syncytial knots; stromal edema and fibrosis	Increased collagen of the villi, intervillar fibrin, fetal endothelial cell proliferation, calcium deposits and edema of the villi			
≥ 14 days				Extensive vascular anomalies in the main villous lumen Extensive fibrosis of the villi	

HISTOLOGICAL MACERATIVE CRITERIA OF BODY FOUND IN WATER

	Motta and Tavani 1980	Janssen 1984
< 20 h	No histological evidence	
≤ 30 h	Conservation of DPN-diaphorasic activities only at vascular and glandular level	
≥ 30 h	Early detachments of the stratum corneum	
≥ 40 h	Cytoplasmic and nuclear alterations of the spinous layer (optically empty perinuclear halos)	
≥ 50 h	Absent DPN-diaphorasic activities	
≥ 72 h	Disappearance of RNA in the spinous layer	
≥ 90 h	Disappearance of the stratum corneum	
≥ 100 h	Partial conservation of the granular layer only; alterations of elastic and reticular fibers	
≤ 7 days		Good stainability of the elastic fibers of the papillary layer
≥ 2-3 weeks		Moderate stainability of the elastic fibers of the papillary layer, without epidermal detachment
≥ 4 weeks		Little or no stainability of the elastic fibers of the papillary layer
≤ 2 months		Preservation of stainability and structure of elastic fibers in lung

3.

RETROSPECTIVE PHASE

As stated in the Introduction, this research was divided into two phases: 1) preliminary retrospective analysis of data, and 2) experimental prospective phase.

The first phase has been designed to bring out and analyze the most critical aspects of the application of various timelines proposed in scientific literature, especially in cases where conflicts have arisen with clinical evidence. The analyzed criteria of scientific literature will then preliminarily tested on a known population in order to understand their real and current validity in daily practice for the diagnosis of fetal death period.

3A. MATERIALS AND METHODS

After completion of the critical review of the literature, a collection and analysis of a selected population was carried out, and then specific micro- and macroscopic attributes were compared in order to make a diagnosis of time of death. This procedure has been used for a comparison "inside" individual cases (occurring, for example, an immediate correspondence between the known time of death and the characteristics of the fetus), in relation to other selected cases and, finally, in comparison with the literature criteria discussed in the previous chapter.

The archives of the neonatal autopsy reports related to events of intrauterine fetal death occurred at Milan San Paolo and Buzzi hospitals during the years 2007,

2008 and 2009 were analyzed, collecting a total of 456 necropsy reports. We used the following selection criteria:

- spontaneous intrauterine death, including cases of twin pregnancies, starting from the 15th week of gestation;
- last survey of vitality, first relief of death and time of the expulsion known and recorded;
- time between the last relief of vitality and the first relief of death not more than 15 days.

Cases related to legal interruptions of pregnancy were deliberately excluded from the study. In such circumstances, in fact, the possibility that the product of conception could be affected by diseases and/or malformations was certainly higher than the general population and this feature, in the view of a global enhancement of the influence of pathologic finding on post-mortem macerative process could have been a bias that we have preferred to avoid. As regards to the fetal vitality and death information, as discussed in previous chapters, they refer only to those actually recorded by ultrasound or other instrumental method, ignoring the data given by the mothers on the moments of presence/absence of perceived fetal movements. Finally, the choice of limiting the period between the last relief of vitality and the first relief of death to 15 days has a twofold explanation: first of all, a time that is too large would have represented a forced dilation of the uncertainty of the time of fetal death, also in consideration of the fact that the maximum waiting time between an ultrasound examination and the next one is generally about a month in physiological pregnancies and even lower in the pathological ones. Also, as was shown by the analysis of the

thanatological criteria in the literature, two weeks is commonly accepted as the maximum limit for the evolution of the post-mortem macerative process, after which the fetus has completely mummified.

For each case, clinical information regarding the pregnancy, the timeof vitality relief, death relief and the time of expulsion were collected; these data were then integrated with the autopsy findings, thus providing a first overview of the pathophysiological and post-mortem features of each case. Subsequently, each case was re-evaluated in a blinded way (ignoring the maximum death-birth time) for three main areas of analysis:

1. evaluation of photographs for the external examination and characteristics of skin color, degree and extent of skin maceration, cranial bones, the color of the serous or blood flows in body cavity;
2. evaluation by light microscopy of histological preparations of individual organs stained with standard Hematoxylin-Eosin staining for the assessment of the conservation, general stainability and, in particular, for the presence of nuclear pyknosis and for the decrease or loss of nuclear basophilia degree;
3. evaluation by light microscopy of histological preparations of placentas and umbilical cord also stained with Hematoxylin-Eosin for the evaluation of the presence of karyorrhexis, leukocyte myocytolysis, main vessel wall and vascular abnormalities in the main villi (endothelial proliferation, fibroblast intraluminal occlusion, diffuse fibrosis, mineralization of the basal membrane).

Each area of analysis was evaluated at different times: first by reviewing all the pictures, then all the histological preparations of the organs and finally those of the placentas. This procedure, although each analysis has already been conducted in a blinded way, made it possible to further reduce the risk of influence of the previous analysis findings in the analysis of individual characteristics.

Regarding histological analysis of organs, it should be noted that the evaluation of the pancreas has always been conducted on the body-tail, never on the head, to avoid the risk of misinterpreting as post-mortem autolytic findings possible artifacts from poor fixation. Issues related to the possible presence of artifacts from poor fixation (and consequent poor stainability) were also observed in presence of acute congestion or extravasation of blood (these aspects will be explored and discussed in section 3c). Still relatively to histological analysis of organs and the placenta, it was decided to organize the results in terms of "focal" and "diffuse" alterations, abandoning the original idea of percentage grading the alterations extent: this criteria, in fact, proved difficult to implement and unintuitive in microscopic observation.

After completion of the data collection, it was decided to organize the subsequent analysis into two separate parts. First of all, all available data were organized to allow internal comparisons of the population, especially as regards to macerative variables related to the death-birth time and the presence of pathological phenomena, and how these can be more or less associated to the progression of post-mortem autolytic phenomena. Subsequently, the results were organized in order to proceed with a comprehensive thanatological evaluation applying to our cases the criteria for diagnosis of time of death discussed in the literature and summarized at the

end of the previous chapter (with particular regard to the work of Genest et al.), this time proceeding with a blinded assessment to the known death-birth times.

3B. RESULTS

After the examination of 456 necropsy reports, 55 cases were selected for the present phase according to the selection criteria outlined above. The clinical characteristics of the selected population are summarized in Table 11.

	Population (55)	Total	%
Week of gestation	Late spontaneous abortion (15-22)	33	60
	Pre-term IUFD (23-36)	18	32,7
	Term IUFD (>37)	4	7,3
Sex	Male	21	38,2
	Female	34	61,8
Pregnancy	Single	31	56,4
	Twin (monochorionic biamniotic)	24	43,6
Associated diseases	Multiple hemorrhages	37	67,3
	Intrauterine infection	17	30,9
	PROM	13	23,6
	Placental abruption	10	18,2
	Hypoxia / ischemia / asphyxia	8	14,5
	Malformations	8	14,5
	Oligo- anhydramnios	8	14,5
	IUGR	7	12,7
	Umbilical vein thrombosis	4	7,3
	Fetal ascites	3	5,4
	Pathological doppler umbilical vessels	3	5,4
	Multiple thrombosis	3	5,4
	Polyhydramnios	1	1,8
	Hydrops	1	1,8
	DIC	1	1,8
	Erythroblastosis	1	1,8
	Prolapse	1	1,8
	Fetal anemia + leukocytosis	1	1,8
	TTTS n.s.	2	3,6
	TTTS 1st stadium	1	1,8
	TTTS 2nd stadium	6	10,9
	TTTS 3rd stadium	7	12,7
	TTTS 4th stadium	4	7,3

Table 11. *Clinical characteristics of the 55 case.*

For the subsequent analysis of the results, the population was stratified according to a chronological principle. It was arbitrarily place the "time zero" (T0) at the time of the relief of death. The next time from T0 to the time of the expulsion, in fact, is usually a waiting technical time required for induction of abortive labor: it is a known time, that only in a few cases is more than 24 hours. But it is to keep into account, since in this period of time the macerative processes advance, being retained in the uterus and amniotic fluid a fetus certainly died. In contrast, the time between the vitality relief and the death relief is very variable and, to different extents depending on the case, it shows a more or less extended degree of uncertainty. However, even where inaccurate, it is a fundamental time as fetal death certainly occurs within this period.

All cases were chronologically stratified bearing in mind that the actual time of death of the fetus was unknown and, therefore, the population was classified by considering the degree of uncertainty as the maximum time of in utero permanence of the died fetus. In the perspective in which the exact time of death of the fetus is not known, the chronological stratification is based on the principle that the "T0-birth" represents the minimum possible time of permanence in the uterus after the death of the fetus (in the extreme case which death occurred precisely at the time of the relief), while the period "Vitality-T0" is, on the contrary, the maximum possible time of permanence of the died fetus in utero (in the limit case, opposite to the previous, in which death has occurred immediately after the last relief of vitality). As the times run from T0, in one sense or another, in the same way increases proportionally the degree of uncertainty of the exact moment of death. The data can therefore be summarized in

an overall "Death-birth Time" numerically harmonizing the various times classes of the population, keeping in mind, for this purpose, the data of the different chronology in the literature discussed in Chapter 2. The final classification is shown in Table 12.

Death-birth T	Cases	%
0	5	9,1
≤ 6 h	1	1,8
≤ 12 h	13	23,6
≤ 24 h	4	7,3
< 1 week	2	3,6
24-36 h	7	12,7
24-48 h	6	10,9
24-72 h	2	3,6
24-96 h	3	5,5
24 h - 1 week	3	5,5
24 h - 2 weeks	4	7,3
48-72 h	4	7,3
48 h - 2 weeks	1	1,8

Table 12. *Final chronological stratification of the 55 cases.*

It was decided not to include the data of birth-autopsy time: on this point, in fact, the scientific literature is unanimous in considering this period of time of no influence on the subsequent post-mortem evaluation of the characteristics of the fetus and placenta, provided that, as in our cases, the fetus and placenta were always kept in a refrigerated chamber.

Table 13 presents the same characteristics of the population in Table 11 divided into the above time classes.

The following Tables 14, 15 (a, b, c) and 16 summarize the main results of the phases of cases observation (respectively: analysis of autopsy reports and observation

of the photographs, histological analysis of organs, histological analysis of placentas) divided into the same time classes.

With regard to Table 14, it should be noted that the "parboiled" appearance of the skin is never mentioned in the autopsy reports analyzed, and in this color category were assimilated the terms "bright red" and "cyanosis rubra", more commonly used. With regards to histological analysis of organs, it has been conducted on a sample of 54 cases as one result was unreadable due to technical problems of inclusion, staining and mounting of the related slides. For the same reason, the histological analysis of the placentas was conducted on a sample of 48 cases. Furthermore, as is apparent from Table 15a, the myocardium was not always found: nevertheless the results when present have been included.

Population (55)		Death-birth Time												
		0	**≤ 6 h**	**≤ 12 h**	**≤ 24 h**	**≤ 1 w**	**24-36 h**	**24-48 h**	**24-72 h**	**24-96 h**	**24h-1w**	**24h-2w**	**48-72 h**	**48h-2w**
Gestational week	Late spontaneous abortion (15-22)	2	1	11	3	2	5	2	1		3	1	1	1
	Pre-term IUFD (23-36)	1		2	1		2	4	1	1		3	3	
	Term IUFD (>37)	2								2				
Sex	Male	3		3	2	1	1	2	1	2	1	3	2	
	Female	2	1	10	2	1	6	4	1	1	2	1	2	1
Pregnancy	Single	5		3	4	2	2	1	2	3	3	4	1	1
	Twin		1	10			5	5					3	
Associated diseases	Multiple hemorrhages	4	1	10	2	1	5	3	1	3	2	2	2	
	Intrauterine infection	1	1	4	3	2	3		1		2			
	PROM	1	1	4	2		3	2						
	Placental abruption	4		2					2			1		1
	Hypoxia / ischemia / asphyxia	1		1			1	1				1	2	
	Malformations			1	1		1	1			2			1
	Oligo- anhydramnios			1		1	1		1		1			
	IUGR				1	1	2	1			2			
	Umbilical vein thrombosis							1	1	1				1
	Fetal ascites							1		1				
	Pathol. doppler umbilical vessels						1	1				1		
	Multiple thrombosis	1								1		1		
	Polyhydramnios									1				
	Hydrops												1	
	DIC				1									
	Erythroblastosis						1							
	Prolapse			1										
	Fetal anemia + leukocytosis									1				
	TTTS n.s.			2										
	TTTS 1st stadium			1										
	TTTS 2nd stadium			4			2							
	TTTS 3rd stadium		1	2			1						3	
	TTTS 4th stadium			1			2	1						

Tabella 13. *Characteristics of the population by time classes.*

Population (55)		Death-birth Time												
		0	≤ 6 h	≤ 12 h	≤ 24 h	≤ 1 w	24-36 h	24-48 h	24-72 h	24-96 h	24h-1w	24h-2w	48-72 h	48h-2w
Skin color	Pink	2		1	1						1			
	Pale	1		2				2		1		1	1	
	"Parboiled" aspect (F)	1			1								1	
	"Parboiled" aspect (D)		1	6	1		2	2	1	1			1	
	Red (F)			2		1	5	1				1	1	1
	Red (D)	1		2	1	1	1	2	1	1	2	1		
	Early mummification											1		
Skin maceration	Absent	4		10	1	1	3	1			1		1	
	Skin flaccidity	1	1	1			1	1	1		1	1	1	
	Bubbles (F)									1				
	Bubbles (D)						1			1		2		
	Epidermal loss (F)			2	2			2		1			1	
	Epidermal loss (D)				1	1	2	2	1	1	1	1	1	1
Cranial bones	Normal	5	1	9	3		1	2			1		2	
	Step			1			3						1	
	More mobility			2		1	2	1	2	3	1	2	1	
	Overlapping			1	1	1	1	3			1	2		1
	Brain colliquation	1				1	1	2					1	
Serous and internal cavities	Smooth and shiny serous	5	1	13	3	2	6	4	1	2	2	1	3	
	Red serous				1			2	1	1		3	1	1
	Plural flow <5 ml			2	1		2	3	1	2	1	2	1	
	Pericardial flow <5 ml			1			1	2		1			1	
	Peritoneal flow <5 ml			2	1		2	1		1		2	2	1
	Multiorgan maceration						1				1			

Table 14. *Analysis of macroscopic characteristics by time classes [F: focal. D: diffuse].*

Population (54)		Death-birth Time												
		0	**≤ 6 h**	**≤ 12 h**	**≤ 24 h**	**≤ 1 w**	**24-36 h**	**24-48 h**	**24-72 h**	**24-96 h**	**24h-1w**	**24h-2w**	**48-72 h**	**48h-2w**
Myocardium	Structure and stainability preserved	5	1	10	2	1	3	1		1	1	1		
	Nuclear pyknosis (F)						1							
	Nuclear pyknosis (D)													
	Decreased nuclear basophilia (F)			1						1				
	Decreased nuclear basophilia (D)										1			
	Loss of nuclear basophilia (F)			1			1	1	1	1				
	Loss of nuclear basophilia (D)							1				2	2	1
Trachea *(epithelium)*	Structure and stainability preserved	5	1	13	4	2	7	6	1	2	2	1	3	
	Nuclear pyknosis (F)													
	Nuclear pyknosis (D)													
	Decreased nuclear basophilia (F)								1	1		1		
	Decreased nuclear basophilia (D)													
	Loss of nuclear basophilia (F)													
	Loss of nuclear basophilia (D)											2	1	1
Trachea *(chondrocytes)*	Structure and stainability preserved	2		7	2		1	1		1				
	Nuclear pyknosis (F)	3	1	5	2	2	5	3		1				
	Nuclear pyknosis (D)			1			1	2	2	1	2	2	3	
	Decreased nuclear basophilia (F)													
	Decreased nuclear basophilia (D)											1	1	
	Loss of nuclear basophilia (F)													
	Loss of nuclear basophilia (D)											2		1
Bronchi *(epithelium)*	Structure and stainability preserved	5	1	13	4	1	6	4	1	3	2	1	3	
	Nuclear pyknosis (F)													
	Nuclear pyknosis (D)													
	Decreased nuclear basophilia (F)					1	1	1				1		
	Decreased nuclear basophilia (D)							2						
	Loss of nuclear basophilia (F)							1	1					
	Loss of nuclear basophilia (D)											2	1	1
Lungs	Structure and stainability preserved	5	1	13	4	1	6	4	1	3	2	1	3	
	Nuclear pyknosis (F)													
	Nuclear pyknosis (D)													
	Decreased nuclear basophilia (F)					1	1	1				1		
	Decreased nuclear basophilia (D)							1	1			1		
	Loss of nuclear basophilia (F)												1	
	Loss of nuclear basophilia (D)											1		1

Table 15a. *Analysis of the histological characteristics of organs by time classes [F: focal. D: diffuse].*

Population (54)		Death-birth Time												
		0	**≤ 6 h**	**≤ 12 h**	**≤ 24 h**	**≤ 1 w**	**24-36 h**	**24-48 h**	**24-72 h**	**24-96 h**	**24h-1w**	**24h-2w**	**48-72 h**	**48h-2w**
Liver	Structure and stainability preserved	5	1	9	1	1	4			1	1			
	Nuclear pyknosis (F)													
	Nuclear pyknosis (D)													
	Decreased nuclear basophilia (F)			2	2	1		1		1	1	1		
	Decreased nuclear basophilia (D)			2			3	3	2				1	1
	Loss of nuclear basophilia (F)						1	2	1					1
	Loss of nuclear basophilia (D)							2		1		3	3	
Pancreas	Structure and stainability preserved	4	1	10	3		2				1			
	Nuclear pyknosis (F)													
	Nuclear pyknosis (D)						1							
	Decreased nuclear basophilia (F)	1		1		1	2							
	Decreased nuclear basophilia (D)			1			2	3	1	2	1	1		
	Loss of nuclear basophilia (F)						1	1				1	1	
	Loss of nuclear basophilia (D)			1				3	1	1		3	3	1
Kidneys *(cortical distal tubules)*	Structure and stainability preserved	4	1	7	1	1	1	1			1		1	
	Nuclear pyknosis (F)			1										
	Nuclear pyknosis (D)													
	Decreased nuclear basophilia (F)	1		3	2		2	1		1	1			
	Decreased nuclear basophilia (D)			2			2	1	1	1			1	
	Loss of nuclear basophilia (F)			3	1		1			1	1		1	
	Loss of nuclear basophilia (D)				1	1	1	3	1	1		4	2	1
Kidneys *(glomeruli)*	Structure and stainability preserved	3	1	12	3	2	3	1	1		1			
	Nuclear pyknosis (F)	2		1			2	2		1		1		
	Nuclear pyknosis (D)				1		2	2	1	2	1	1	3	
	Decreased nuclear basophilia (F)									1				
	Decreased nuclear basophilia (D)											1	1	
	Loss of nuclear basophilia (F)													
	Loss of nuclear basophilia (D)							1				2		1
Kidneys *(other structures)*	Structure and stainability preserved	5	1	13	4	2	7	4	2	2	2	1	3	
	Nuclear pyknosis (F)													
	Nuclear pyknosis (D)													
	Decreased nuclear basophilia (F)									1				
	Decreased nuclear basophilia (D)							1				1	1	
	Loss of nuclear basophilia (F)													
	Loss of nuclear basophilia (D)							1				2		1

Table 15b. *Analysis of the histological characteristics of organs by time classes [F: focal. D: diffuse].*

Population (54)		Death-birth Time												
		0	≤ 6 h	≤ 12 h	≤ 24 h	≤ 1 w	24-36 h	24-48 h	24-72 h	24-96 h	24h-1w	24h-2w	48-72 h	48h-2w
Adrenal glands *(mature cortex)*	Structure and stainability preserved	4	1	7	1	1	3			1	1		1	
	Nuclear pyknosis (F)													
	Nuclear pyknosis (D)													
	Decreased nuclear basophilia (F)	1		5	2			2			1	1	1	
	Decreased nuclear basophilia (D)			1	1	1	4	3	1	1				
	Loss of nuclear basophilia (F)				1			3	1	1				
	Loss of nuclear basophilia (D)								1	1		3	2	1
Adrenal glands *(other structures)*	Structure and stainability preserved	5	1	13	4	2	7	6	1	2	2	1	3	
	Nuclear pyknosis (F)													1
	Nuclear pyknosis (D)													
	Decreased nuclear basophilia (F)									1				
	Decreased nuclear basophilia (D)			1					1			1	1	
	Loss of nuclear basophilia (F)													1
	Loss of nuclear basophilia (D)											2		
Duodenum / intestine	Structure and stainability preserved	4	1	9	1	1	3			1		2		
	Nuclear pyknosis (F)				1									
	Nuclear pyknosis (D)										1			
	Decreased nuclear basophilia (F)	1		2	1		1							
	Decreased nuclear basophilia (D)			1		1	3	3	1	1	1			
	Loss of nuclear basophilia (F)			2				1		1			1	
	Loss of nuclear basophilia (D)							2	1	1		2	3	1
Colon/ Rectum	Structure and stainability preserved	5	1	10	1		2			1	1			
	Nuclear pyknosis (F)					1								
	Nuclear pyknosis (D)													
	Decreased nuclear basophilia (F)			1	2		2							
	Decreased nuclear basophilia (D)			1		1	2	3	1	1	1		2	
	Loss of nuclear basophilia (F)			2	1		1	2		1	1	2	1	
	Loss of nuclear basophilia (D)							2	1	1		2	1	1
Skeletal muscle	Structure and stainability preserved	5	1	10	4	1	6	4	1	1	2	1	3	
	Nuclear pyknosis (F)													
	Nuclear pyknosis (D)													
	Decreased nuclear basophilia (F)			2		1	1	1		2				
	Decreased nuclear basophilia (D)			1				1	1			1	1	
	Loss of nuclear basophilia (F)							1				1		1
	Loss of nuclear basophilia (D)											1		

Table 15c. *Analysis of the histological characteristics of organs by time classes [F: focal. D: diffuse].*

Population (48)		Death-birth Time												
		0	**≤ 6 h**	**≤ 12 h**	**≤ 24 h**	**≤ 1 w**	**24-36 h**	**24-48 h**	**24-72 h**	**24-96 h**	**24h-1w**	**24h-2w**	**48-72 h**	**48h-2w**
Leukocyte karyorrhexis	Absence of karyorrhexis	2	1	3		1	1							
	Umbilical cord vessels (F)			1	3	1	4	1	1	2	1	1	3	
	Umbilical cord vessels (D)			1			1	1			2	2	1	1
	Main villi vessels (F)			8	2		4	3	1	2	1	2	1	
	Main villi vessels (D)				1	1	2	3	1		2	1	3	1
Myocytolysis	Absence of myocytolysis	1	1	5	1						1			
	Umbilical cord vessels (F)			3	2	1	1	1	1	2				
	Umbilical cord vessels (D)	1		1		1	5	5			2	3	4	1
	Main villi vessels (F)	1		3	2	1	6	4	1	2	2	1		1
	Main villi vessels (D)			1	1	1	1	2	1			2	4	
Vascular abnormalities in main villi	Absence of anomalies	2	1	10	3	1	3	3		2	1	1		
	Endothelial proliferation (F)			1	1				1		2	1	1	
	Endothelial proliferation (D)						2	2					1	
	Intraluminal fibroblasts (F)				1	1	2	1	1		1	2	1	
	Intraluminal fibroblasts (D)							1					3	1
	Complete lumen occlusion													
	Diffuse villous fibrosis													
	Mineralization basal membrane (F)				1		1	1					3	
	Mineralization basal membrane (D)													

Table 16. *Analysis of microscopic placental features by time classes [F: focal. D: diffuse].*

3C. DISCUSSION

The characteristics of the study population and the results achieved will be first discussed and, subsequently, these results will be compared with data from the scientific literature analyzing any differences and concordances.

The characteristics of our population are summarized in Tables 11, 12 and 13. As expected, most cases are intrauterine deaths occurred before the 23rd week of gestation (60%), when, in fact, such clinic events are expected with more frequency. In this population there are 24 cases (43.6%) of twin pregnancies, during several weeks of gestation. Clearly, these cases present anatomical, clinical and pathological characteristics very particular, so as to merit a separate discussion. However, at this stage of progression, it was decided to include them in the analysis without distinction and the study was conducted without it being possible to recognize twin in the course of macroscopic and histological analysis. The rationale for this choice is based on the fact that twin pregnancy was never considered differently from the single one in the discussion pf post-mortal modifications in fetal death. In other words, the anatomical and clinical particularities that characterize this type of pregnancy (and that inevitably characterize even the death and thieir related phenomena) have never been used to differentiate them in the study of macerative chronological trend, contrary to certain diseases. Therefore, felt obliged at this stage to uniform our data to allow a comparison with the literature, we have chosen not to discuss twin pregnancies separately from single ones. It should be noted, still on the subject of twin pregnancies, that they represent paradigmatic cases for the purpose of fetal thanatology understanding: in our

experience it is not uncommon to see cases of twins who die and are expelled virtually the same time but exhibiting macerative characteristics entirely different. The bases for these differences are likely to be sought in the tipycal disases of twin pregnancies (above all, the Twin-to-Twin Transfusion Syndrome - TTTS -) and how their presence may affect post-mortem phenomena in terms of chronology or in possible interference of particular therapies (for example, the laser-therapy).

With regard to the various conditions associated with pregnancy, it was decided to include in the discussion anything that deviates from a normal pregnancy. Again, the reasons lie in the structuring of the studies in literature: it was never given a real statistical weight or even epidemiological value to fetal and placental pathological variables, so it was not possible to decide in advance what to include and what to leave out in the present study.

Relatively to chronological stratification of the population, it is worth highlighting as in most cases death occures in a period comprised within 24 hours prior to its actual instrumental survey: among the 55 study cases, in fact, in 23 (42%) death can be traced back to the last 24 hours. Similarly, since the relief instrumental of death, 89% cases are expelled within 24 hours. From these data it is clear that we often discuss a relatively short period of time, during which it is reasonable to assume a very low possibility of differentiation based on chronological macroscopic or histologic criteria. It is precisely on the basis of these limitations that will be set the second phase of the study.

The macroscopic analysis was conducted by evaluating the autopsy reports and observing the photographs of individual cases, and the results are summarized in Table 14. Reading the table horizontally for each of the four areas analyzed (skin color, skin maceration, state of the cranial bones, serous and internal cavities), the presence of a common "downward trend" can be observed. In other words, in the first columns prevail cases placed in high boxes (early or absent changes), while in the last column prevail those located in the lower boxes (widespread or advanced modifications). This "at first glance" aspect (also present in the results of histological investigations), lead to considered as correct the criteria chosen for this analysis, as a grossly correlation between their advancement and the uterine retention time after fetal death do exists.

However, carefully analyzing the results and moving from a horizontal to a vertical reading of the table, it can be seen see that despite the common downward trend mentioned before each column never appears to be homogeneous. If this general dispersion of the results is just what we were waiting for larger time classes (for example, the classes "24h-1w" or "24h-2w"), it was more difficult to predict the presence in the class "0" of fetuses with "parboiled" skin (1 case) or even diffusely red skin (1 case), with colliquated brain (1 case) or even with a generalized grade I maceration (1 case). In the third column, corresponding to the time class "≤ 12 h", this aspect is even more pronounced: although the majority of the cases presented absence of maceration (10 cases) and a normal state of the cranial bones (9 cases), there are also cases with focal grade III macerative areas (2 cases) or a complete loss of relations of cranial bones (1 case). Similarly, observing the column "48-72 h", cases with absence of skin maceration (1 case) and normal state of the cranial bones (2 cases) can still be

found. The major dispersions of results are shown in columns "24-36 h" and "24-48 h", where all the variables involved are shown.

Some qualitative considerations can be done in order to try to motivate these results.

The **skin color** can be altered and influenced by three main factors: gestational age, presence of disease, maceration degree. Premature infants, in fact, show a physiological more reddish skin color, due to the greater thinness of the skin and the presence of fetal hemoglobin in the blood, and this factor can be confused with a "parboiled" aspect or with a diffuse red macerative color, although the color can be normal for the gestational age. Therefore, from a descriptive point of view, the color of the premature fetus as "parboiled" or red is properly framed, but from an interpretative point of view must be considered that this is a physiological variable and not necessarily related to macerative post-mortal phenomena. Similarly, the presence of diseases can greatly confuse the color of the skin: for example, there are many difference between a plethoric receiver TTTS fetus or a fetus died as a result of extensive hemorrhaging, who will appear with a deep red color, and an anemic or a TTTS donor fetus, who will look pale, regardless of the degree of maceration. Even in this case is necessary to distinguish the description of skin color from its interpretation, trying to recognize the cases in which the disease, and not the post-mortal transformations, caused a modification of the color of the skin. Finally, the macerative processes, which proceed according to a known sequence, overlap with pre-existing conditions. This is important when you consider that most of the intrauterine deaths involv immature fetuses or those with disease or conditions associated with both.

As regards to the phenomena of **skin maceration**, probably what most influences its temporal trend over the uterine retention time is, once again, the presence of diseases. As already reported in the literature, the amount of internal or external liquid greatly influences skin maceration, this being a process of imbibition and autolysis: where imbibition is greater (polyhydramnios, hydrops, ascites) post-mortem phenomena are accelerated, while in cases of oligohydramnios or anhydramnios maceration results slowed. In addition to these intuitive and overall correct considerations, in our experience the single pathological condition that has most affected the progression of macerative phenomena was chorioamnionitis: where there was a septic fetus the degree of maceration was almost constantly greater than expected for the corresponding temporal class. The same considerations, although with a lesser degree, can be made for the presence of fetal extensive hemorrhaging. Again, the description of the macerative degree will always be correct, but its chronological interpretation will be affected by the underlying pathology. Another confounding factor is represented by the possible action of mechanical-traumatic forces on the skin during the expulsive phase or in the course of the successive manipulation of the corpse. Traumatic force can act in different ways: it can cause breakage of post-mortem dermo-epidermal bubbles, confounding a grade II maceration with a grade III, or it can directly cause a focal de-epithelialization, especially in areas of support and where the skin is more fragile, mimicking a grade III maceration. In the latter case, however, the remaining skin areas are intact and free of post-mortem transformations. In this case will be incorrect the description of the phenomenon even before its interpretation, as etiologically different.

Coming now to the **state of the cranial bones**, it is an extremely variable and easily alterable condition from pre- and post-mortem circumstances, as can be seen from the significant dispersion of results. The most important of these conditions is certainly the conservation status of the brain. Most often, in fact, the brain colliquates very quickly after death, even in a few days, thereby causing the bones of the skull to be unstable and hypermobile, thus leading to highlight the loss of connection between the cranial bones where it is instead an "indirect" condition, not linked to macerative skin phenomena but to the internal condition of the brain. The same consideration, but reversed, can be made in the case of massive cerebral edema, which presence can initially postpone the moment at which it will be evident the presence of a step or high mobility of the cranial bones. Similarly, a great imbibition of the scalp will lead to greater distension of the soft parts with a normal appearance of the cranial bones prolonged in time. A proper assessment of the state of the cranial bones may be therefore made only after assessing the state of the brain, but in this context it will be unlikely to be able to modulate the chronological judgment, because once exposed the cranial cavity and highlighted the state of the brain, it will be no more possibile to define how the detection of a colliquative condition or an intense edema have changed the mobility of the cranial bones, making this criterion often unreliable.

Finally, the evaluation of **serous and internal cavities** in terms of color, flows presence and multiorgan maceration is greatly affected by pathological variables, creating in this case also a significant dispersion of the results: in the results table, in fact, this variable is the one that has a lower downward trend, constantly showing cases with smooth and shiny serous even after several days and where the other post-

mortem maceration features and the state of the cranial bones would lead to a greater chronological judgment. In our experience, this is the least reliable data on which to base the assessment of the death-birth time.

The results of the analysis of histological features of the organs, in terms of nuclear characteristics and stainability, are summarized in Tables 15a, 15b and 15c. Even in these tables, as for the macroscopic analysis, a general downward trend in all organs examined can be see, confirming the intuitive goodness of the chosen criteria. In particular, the evaluation of the decrease or loss of nuclear basophilia, as the direct observable expression of autolytic phenomena, has been very reliable, although not in all organs in equal measure. As deduced also by Genest et al. in their studies, the organs that show a better correlation between the degree of autolysis and the death-birth time are the liver, the kidneys (cortical distal tubules), the adrenal glands (mature cortex) and the gastro-intestinal tract, to which, in our experience, we can also add the pancreas: although the results are not always consistent, these organs showed degenerative patterns in terms of nuclear stainability well correlated with the intra-uterine retention time, suggesting that further efforts in this direction can reserve also important applicative results. Also in terms of "vertical" reading of the tables and degree of dispersion of the results, the organs in which the characteristics of nuclear stainability showed lower correlation turn out to be the tracheal epithelium, lungs and other kidney and adrenal gland structure. The remaining organs showed an intermediate degree of correlation.

In this study was evaluated also nuclear pyknosis in terms of presence and extension: this characteristic, although known and typical of post-mortem autolytic phenomena, has never been correlated with the fetal tanato-chronological judgment. In our experience, at least two organs showed interesting results: trachea (chondrocytes) and kidneys (glomeruli). While the presence of focal nuclear pyknosis can be detectable in all organs at different chronological moments (even at the time "0"), the extensive presence of nuclear pyknosis is never present in a uniform manner prior to 24 hours in tracheal chondrocytes and in glomerular cells. Even in this case, a deepening in this sense could reserve useful results.

A very important consideration regarding the histological analysis of organs is the possible source of confounding reading. As already mentioned in the previous chapters, the level of stainability of a tissue, regardless of its source, is related to the fixation degree achieved by the tissue itself after permanence in formalin. If the tissue is poorly fixed or there are portions that have acquired less fixative, the result will be an attenuation of both the acidophilia and basophilia of the tissue. Such cases is often present in pancreas head, usually colored in paler shades compared to body and tail, and for this reason the reading of the pancreas has never been conducted on the head portions. In the same way act the presence of bleeding or severe congestive states: such conditions retard the fixation of the tissue that consequently is less effective in acquire the color. Although these occurrences are commonly known in histo-pathological practice, it must be remembered and kept in mind where a judgment concerning the observation of histological sections is based essentially on the level of tissue staining. It

would be a bad mistake to confuse fixation artifacts with post-mortem macerative processes.

Finally, one last consideration on histological analysis of the intestinal tract. During the reading of the slides, it was noted often a "focal" loss of nuclear basophilia limited to the inner portion of the wall directly in contact with meconium (epithelium of the villi and crypts), accompanied by a perfect preservation of the stainability of other structures. In these cases, classified as "decrease/focal loss", it is perhaps possible to hypotize a direct chemical action of meconium on mucous membranes. If in doubt whether this finding is attributable to chemical degeneration rather than post-mortem maceration, it might be advisable to evaluate only the outer part, leaving out all the mucous membranes directly in contact with meconium.

The results of placental histology are summarized in Table 16. Once again it is possible to trace a downward trend in a horizontal reading of the table and a more or less important vertical dispersion of the results.

It should be said that the placenta is a very variable organ that in the course of pregnancy, especially in the terminal stages, physiologically shows a series of degenerative phenomena. It is important that these are clearly distinct from post-mortem maceration and in this sense must be read the gradual abandonment in literature of the evaluation of syncytial knots or mineralization of the basal membrane of the main villi (the latter also negatively evaluated in the present study, completely lacking correlation with death-birth time): it is necessary to completely exclude these criteria from a proper assessment of the time of fetal death.

The two characteristics that in our experience have proved to be greatly related to the death-birth time are the presence of leukocyte karyorrhexis in the vessels of the umbilical cord and main villous and myocytolysis of the umbilical cord vessel wall. While the leukocyte karyorrhexis in the vessels of main villi is observable from the first hours and remains present in all time classes (although more detectable after 24 hours), the leukocyte karyorrhexis in the vessels of the umbilical cord is, instead, later, with a full manifestation generally beyond 24 hours. Widespread myocytolysis in vessel wall of umbilical cord is consistently highlighted after 24 hours of intrauterine retention. Myocytolysis of the villi and the presence of major vascular abnormalities in the villi have proved to be less reliable. In fact, in recent literature is reported as vascular alterations in the villi are present in extended form only after many days since death (up to 4 weeks), whereas in the present study were never exceeded two weeks of uterine retention: complete lumen occlusion or diffuse villous fibrosis were never observed.

Because of the great variability of placental criteria, the correlation between the clinical-pathophysiological data and the post-mortem maceration is complex. The placenta, in fact, react very differently from the fetus to external insults and in a less linear way: even in the case of an extensive chorioamnionitis, where maceration appeared generally accelerated, the placental finding proved to be often constant, albeit anatomically altered.

COMPARISON WITH THE LITERATURE

After discussing the characteristics of the population studied and the results of macro- and microscopic observations, it is essential to proceed with what represents the node of the first phase of this study: the comparison with the criteria of scientific literature. In particular, it is necessary to verify the degree of correlation of the death-birth time of all the cases on the basis of several thanatological criteria previously discussed in the literature (see summary tables at the end of Chapter 2).

By applying the criteria proposed by the analyzed authors, each case has been placed in a "death-birth" time range and later the real correspondence with the chronological stratification used to divide our cases was verified (Table 12).

The results of this analysis, expressed as percentage of correct correspondence, are shown in Tables 17 and 18 relating, respectively, to the macroscopic and histological analysis of organs and histological placental analysis.

The first and foremost consideration is the percentage of total correspondence between the studies. Among the various chronology about maceration and fetal histology (Table 17) a correspondence higher tan 80% was never observed: the two works by Genest et al. have been those that have allowed a greater accuracy in the placement of our study cases (corresponding to 80 and 76%). The chronology proposed by Maroun et al. has shown the lower correspondence (45%). As regards to the criteria of fetal histology, once again the criteria of Genest et al. have allowed a greater correspondence (81%), while in the worst case it has never fallen below 69%.

Death-birth Time	Langley 1971	Bain 1974	Wiggles. 1984	Singer 1991	Genest 1992, I*	Genest 1992, III	Maroun 2005
0	80% (4)	80% (4)	80% (4)	80% (4)	100% (5)	80% (4)	80% (4)
≤ 6 h	100% (1)	100% (1)	100% (1)	100% (1)	100% (1)	100% (1)	100% (1)
≤ 12 h	85% (11)	85% (11)	69% (9)	77% (10)	85% (11)	85% (11)	69% (9)
≤ 24 h	50% (2)	50% (2)	50% (2)	50% (2)	100% (4)	100% (4)	25% (1)
< 1 week	100% (2)	100% (2)	100% (2)	100% (2)	100% (2)	100% (2)	50% (1)
24-36 h	14% (1)	71% (5)	14% (1)	14% (1)	43% (3)	29% (2)	14% (1)
24-48 h	50% (3)	67% (4)	17% (1)	17% (1)	83% (5)	67% (4)	17% (1)
24-72 h	50% (1)	50% (1)	100% (2)	50% (1)	100% (2)	50% (1)	50% (1)
24-96 h	100% (3)	100% (3)	100% (3)	67% (2)	67% (2)	100% (3)	33% (1)
24 h - 1 week	33% (1)	67% (2)	33% (1)	67% (2)	33% (1)	67% (2)	0
24 h-2 weeks	100% (4)	100% (4)	100% (4)	100% (4)	100% (4)	75% (3)	75% (3)
48-72 h	75% (3)	50% (2)	100% (4)	75% (3)	50% (2)	100% (4)	50% (2)
48 h-2 weeks	100% (1)	100% (1)	100% (1)	100% (1)	100% (1)	100% (1)	0
Total	**67% (37)**	**76% (42)**	**64% (35)**	**62% (34)**	**80% (43)**	**76% (42)**	**45% (25)**

(*) analysis conducted on 54 cases

Table 17. *Correspondence between the thanatological macroscopic and histological criteria in literature and the temporal stratification of the 55 cases studied.*

Death-birth Time	Fox 1968	Wiggles. 1984	Silver 1988	Genest 1992, II	Jacques 2003
0	100% (2)	100% (2)	100% (2)	100% (2)	50% (1)
≤ 6 h	100% (1)	100% (1)	100% (1)	100% (1)	100% (1)
≤ 12 h	91% (10)	91% (10)	91% (10)	91% (10)	64% (7)
≤ 24 h	75% (3)	75% (3)	75% (3)	75% (3)	75% (3)
< 1 week	100% (2)	100% (2)	100% (2)	100% (2)	100% (2)
24-36 h	86% (6)	43% (3)	43% (3)	43% (3)	43% (3)
24-48 h	83% (5)	50% (3)	50% (3)	50% (3)	100% (6)
24-72 h	100% (2)	0	0	100% (2)	100% (2)
24-96 h	0	100% (2)	100% (2)	100% (2)	100% (2)
24 h - 1week	67% (2)	100% (3)	100% (3)	100% (3)	67% (2)
24 h-2 weeks	67% (2)	100% (3)	100% (3)	100% (3)	100% (3)
48-72 h	25% (1)	0	0	100% (4)	100% (4)
48 h-2 weeks	100% (1)	100% (1)	100% (1)	100% (1)	100% (1)
Total	**77% (37)**	**69% (33)**	**69% (33)**	**81% (39)**	**77% (37)**

Table 18. *Correspondence between the thanatological placental histological criteria in literature and the temporal stratification of the 48 cases studied.*

These results demonstrate the greater effectiveness of criteria derived from a solid scientific analysis (those of Genest et al.) as compared to other more empirical ones based exclusively on the direct observation of the authors. But at the same time cannot be negatively highlighted the mismatch: at best, about 20% cases that escape by the existing schedules remains, which show macerative and autolytic characteristics so different from those expected that cannot be properly evaluated. Furthermore, it should be added that among the cases with less general correlation are those suffering from conditions such as TTTS in case of twins, of excess or deficiency of amniotic fluid and especially infections.

The most general successful, in terms of total matching, of fetal criteria compared to placental criteria (69-81% compared to 45-80%) can not take into consideration the fact that placental thanatology consider less variable, with timing generally less strict, allowing a wider temporal placement in classes. In table 18, in fact, many time classes are witnessing a "all-or-nothing" phenomenon, where none or 100% of the cases are correctly classified ("24-72 h"; "24-96 h"; "48-72 h"): this is the result of the application of very broad criteria, which are not discriminating intermediate times. The closer correspondence is then offset by a lower overall accuracy.

Considering more in detail the percentages of correspondence among individual time classes, the highest percentages are present in the early stage (under 12 hours) or in the very broad times ("24h-2 weeks", "48h-2 weeks"), albeit with clearly distinct meanings: in the first case there is a real precise correspondence, while in the latter there is a correct but inaccurate classification. In both tables (fetus and placenta) the

time classes which recorded a lower correspondence are those of "24-36 h" and "24-48 h", with rates as low as 14% agreement.

Ultimately, it seems that it is possible to indicate with reasonable accuracy if the fetus has been dead since a few hours (within 12-24 hours) or has been dead for a longer time (1-2 weeks), while successfully graduate intermediate timeframes is still very difficult because of the presence of imprecise criteria or for their complete absence. Also lacking among these criteria the possibility of an adjustment in the chronological order based upon the presence of specific fetal or placental diseases.

4.

PROSPECTIVE PHASE

4A. PREPARATION OF THE STUDY

After the results of the retrospective phase, which have highlighted the limitations of existing chronologies in the literature, it was decided to focus the attention for the second part of the study to the most critical phases of death-birth time. From the comparative data of our cases with literature has emerged that the application of the various chronologies is partially effective to indicate whether the fetus has been dead for a few hours (within 12-24 hours) or for a long time (1-2 weeks), while successfully graduate intermediate timeframes is still very difficult. However, focusing on the early times and carefully observing the results of the retrospective phase and the summary tables at the end of Chapter 2, it is clear that the earliest thanatological criteria is hardly be able to distinguish the intermediate timeframes below 12 or even 24 hours. In other words, even where it can be fairly reliable the placement of the death of the fetus in a period "below 12-24 hours", there is no reliable criteria for defining shorter time intervals and any macroscopic or histological characteristic was never observed below 4-6 hours.

Taking into account that the critical period more often involved in forensic litigation (which is the ultimate purpose of this study) is represented by the first few hours after death, we have decided to concentrate the second phase of the study in this time interval.

Among the possible innovative orientations acts to overcome the limitations of optical microscopy, the morphological approach was the primary choice for greater consistency and continuity of the project, and for greater accessibility to means and resources. In particular, it was decided to use the transmission electron microscopy (TEM) to operate a morphological analysis of the possible stages of cell organelles degeneration positively correlated to the time of death. As expected, the main difficulty of the experimental perspective was the choice of the study population, since within any possibility of standardization there was the necessity to use only those cases where the time of death was precisely known. In this sense, the voluntary interruption of pregnancy in the first three months would have been a choice with guaranteed levels of reliability of the time of death (known as mechanically induced with surgery) and availability of samples.

However, a problem of normative character for a chance to take biological samples in fetal abortions does exist.

In Italy it is required by art. 74 of the Regio Decreto 09.07.1939 n. 1238 the obligation to register all births (fetuses who have successfully completed 28 weeks of gestation at birth) with the consequent right to the burial and the funeral service. In case of abortion, Italian cemetery regulations, with local variations, are based on the DPR 10.09.1990 n. 285, according to which the burial of fetuses dead before 28 weeks and even of aborted fetuses for any reason or under any circumstances (including the voluntary interruption of pregnancy) can be done if the parents request it. The Law sets no limit of gestational age below which the burial of the child cannot be done. In Lombardy Region the Regolamento Regionale 09.11.2004 n. 6 has modified the

current Regolamento Regionale 06.022007 n. 1 (Regulation concerning funeral and cemetery activities) by introducing a duty on the hospital operator to inform parents of the opportunity to ask for the burial, and, in the absence of a request by the parent, the obligation of the Lombardy Region to provide however to the burial of the fetus, regardless of gestational age. It is required that these provisions may be waived only in the presence of reasons of diagnostic character: in other words, if a need arises to autopsy the fetus or even a simple histological analysis of tissue fragments, it is possible to overcome the mandatory burial, even after the analysis is completed. However, this scientific project wouldn't allow to take advantage of the exemption provided in regional regulations, not having subsisting diagnostic reasons to perform a voluntary abortion in the first 90 days.

Therefore, given the legislative framework, the cooperation of hospitals was subject to the research project approval by the respective ethics committees. Being an inter-departmental research (involving the Section of Legal Medicine, the Department of Human Morphology and the Departments of Pathology and Obstetrics-Gynecology of San Paolo Hospital) was initially sought the opinion of the Ethics Committee of the Milan University, which was performed on the research project and a model of patients informed consent. The project was approved on July 19th 2012 with approval of the Committee, after a change to the consensus model initially proposed (the model of consensus that has been sign by voluntary patient is attached at the end of this chapter). Subsequently, the research project was also submitted to the Ethics Committee of the San Paolo Hospital for final approval, obtained on October 26th 2012.

4B. MATERIALS AND METHODS

As already said, it was decided to consider only the population of voluntary interruptions of pregnancy, excluding for this stage the population of therapeutic abortions of the second and the third quarter, being therapeutic abortions induced by the presence of malformations or other diseases and intrauterine deaths burdened by the uncertainty of the exact moment of death. The main limitation of the choice is represented by the different maturation of fetal tissue in the first quarter compared to a fetus at the end of pregnancy, which is the purpose of this study. For this reason, we chose to use a fetal population as much mature as possible to the extent permitted in the first quarter: cases of abortions during the tenth to twelfth week of gestation.

The collection of biological material coming from voluntary interruptions of pregnancy (after obtaining the consent of the women) was performed at the Unit of Obstetrics and Gynecology, San Paolo Hospital in Milan. Each sampling was limited to the skin and the skeletal muscle of upper and lower limbs, in order to perform the sampling of structures easily macroscopically identified with a single pickup, allowing also to limit any artifacts from elastic retraction of the sample. It was therefore developed an experimental macerative protocol, conducted by placing each piece in an appendorf containing saline solution (Baxter Sodium chloride 0.9% solution for infusion) as a surrogate of the amniotic fluid in the first quarter. Each appendorf was maintained in flotation in a thermostated bath at 37-38°C for a predetermined period of time, after which the saline solution was replaced with the relative fixative medium. Each sample has been divided into 18 frames: nine were fixed in 10% neutral buffered

formalin for optical microscopy analysis and the remaining were fixed for electron microscopy analysis by 2.5% glutaraldehyde in 0.13 molar phosphate buffer at pH 7.2-7.4. As programmed, for each sample was then performed a morphological analysis by TEM and a comparative optical microscopy to each of the nine times programmed, for identification of any sub-cellular organelles degenerations positively correlated with the time of death. The preparation for the optical microscope has provided the usual post-fixative techniques and staining with standard Hematoxylin-Eosin. For the TEM analysis the samples were post-fixed in a 1% OsO4 solution, dehydrated in ethanol and propylene oxide and then embedded in epoxy resin. Ultrathin 50-60 nm sections were then stained with uranyl acetate and lead citrate. The analysis was conducted using a Jeol JEM 1010 electron microscope (Tokyo).

In Table 19 is reproduced the study protocol for the sampling and analysis of the experimental phase.

It should be specified that the "Time 1", for technical and organizational reasons, does not correspond exactly to the time of death of the fetus: the time to complete surgical extraction, transport to the sampling room, identification of the anatomical structures and preparation of 18 samples has imposed a deviation from the time of the death of about 2-3 minutes.

It should be noted, also, that the last sampling (T9) was placed beyond the time of interest for this study with the sole purpose of favoring the appearance of items related to tissue degeneration.

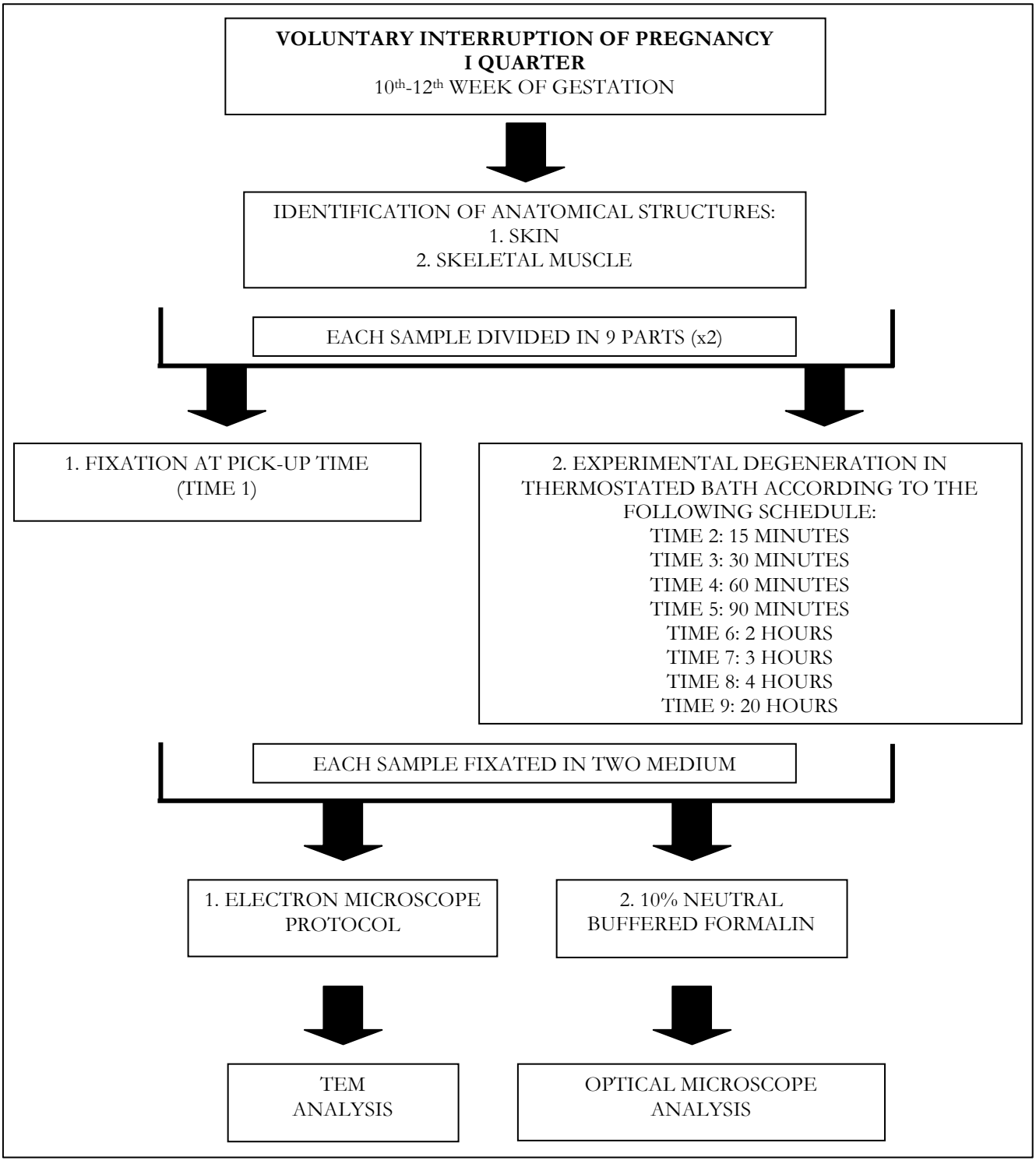


Table 19. *Experimental protocol of the perspective phase.*

4C. MORPHOLOGY OF FETAL SKIN AND MUSCLE

Since cellular and sub-cellular fetal morphology of skin and muscle is significantly different in the first quarter compared to the fetus at term pregnancy and adult subject, it is worth briefly pausing in the exposition of their features to better understand the reading of microscopic preparations exposed in the following section.

The epidermis at the end of the second month of pregnancy (8 weeks) is composed of only two layers of cells: a germinative layer and a surface layer or periderm. During the third month (9-12 weeks) becomes progressively evident a third intermediate layer between the germinal layer and the periderm. At 10 weeks the glycogen is always abundant in cells of both layers; at 11 weeks glycogen is less evident in the cells of the basal layer, but larger amounts are present in the periderm and in the intermediate layer. The cells of the periderm are crushed to the surface, and a few apical microvilli become apparent from week 10^{th}-11^{th}. Neighboring cells are joined by junctional complexes, which at this age can be considered as fully developed desmosomes. At 10-11 weeks intracytoplasmic organelles are also well developed (Hoyes 1968). The epidermis of a fetus at 12 weeks consistis of a basal germinative layer, one to three intermediate layers and a surface layer (periderm). Germinative layer consists of small round or oval cells densely grouped, with a rather dense cytoplasm, anchored to the basal membrane. The cytoplasm is very dense due to the presence of glycogen in the intercellular spaces. The cells contain few tonofilaments but many organelles such as mitochondria and Golgi complexes (compared to those of the more superficial layers). At this stage there are no melanocytes in the epidermis in Caucasoid subjects; rather dermal melanocytes are present along the dermal capillaries. The cells

of the intermediate layer show a greater cytoplasmic volume (up to twice) and glycogen is present in larger amounts in the perinuclear cytoplasm; tonofilaments are noticed from desmosomes. In the surface layer (periderm) cells still contain a large amount of glycogen, abundant tonofilaments and partially degenerate organelles. Plasmatic membranes are still joined by many desmosomes of normal appearance; plasmatic membrane of the free surface is thicker and shows numerous microvilli, covered by a layer of electron-dense material in filamentous structure, very scattered mitochondria and poorly evident Golgi complex (Breathnach and Wyllie 1965, Hashimoto et al. 1966).

Regarding striated muscle anatomy, embryonic development goes through the stages of myoblast, myocyte and myotube: myotube are the stage previous to the development of mature muscle fibers.

At 10 weeks of gestation muscle cells with "myotube satellite cells" occurred in groups which were often large. The cells contained relatively few myofilaments and were often rich in glycogen. Blood vessels and connective tissues generally were sparse and no motor nerve endings were seen. Muscle cells could be identified by their content of myofilaments set in characteristic hexagonal arrays. Muscle cells ranged from 3x5 μm to7x8 μm when transected through the nuclear region; the nuclei were sometimes centrally, sometimes peripherally placed within the cells, apparently independently of cell size. A Golgi apparatus was often conspicuous close to the nucleus and mitochondria were found in all parts of the cytoplasm except where glycogen was present; lipid inclusions were also occasionally seen. Although rough endoplasmic reticulum was present in small quantities, neither smooth endoplasmic

reticulumn or transverse tubules were identifiable at this stage. "Myotube satellite cell" cytoplasm was generally of moderate electron density and contained a Golgi apparatus, a few mitochondria and a little granular endoplasmic reticulum; centrioles and a cilium were occasionally seen. A basal lamina was not present in association with these cells.

At 12 weeks motor end plates were frequently seen, but sensory nerve endings were not recognized. Blood vessels and connective tissues were much more conspicuous than in the smaller specimen and basal laminae were present on muscle cells as well as on the cells of blood vessels. The muscle cells of this specimen, although of similar calibre to those in the younger specimen, seemed generally to be more densely packed with myofilaments, so that glycogen, when present, occupied only a small part of the cross sectional area of a cell. Nuclei were sometimes centrally and sometimes peripherally located, apparently independently of cell diameter. Cells containing only a few myofilaments were extremely rare. Some muscle cells were of extreme electron-density. "Myotube satellite cells" were numerous in this specimen, sharing basal laminae with associated muscle cells, and provided with extensive cytoplasmic processes. Some "myotube satellite cells" were of moderate electron density and contained a Golgi apparatus, granular endoplasmic reticulum, centrioles and cilium as in the younger specimen; others were of very extreme electron density and were identified by their form and by their basal lamina shared with muscle cells. (Bourne 1960; Gamble et al. 1978).

4D. RESULTS

We try to obtain a population as homogeneous as possible for patient age, absence of known diseases and gestational age. Between March and May 2013 four selected women gave their consent for the study and were marked with anonymous A → D letters for privacy reasons. They present the following characteristics:

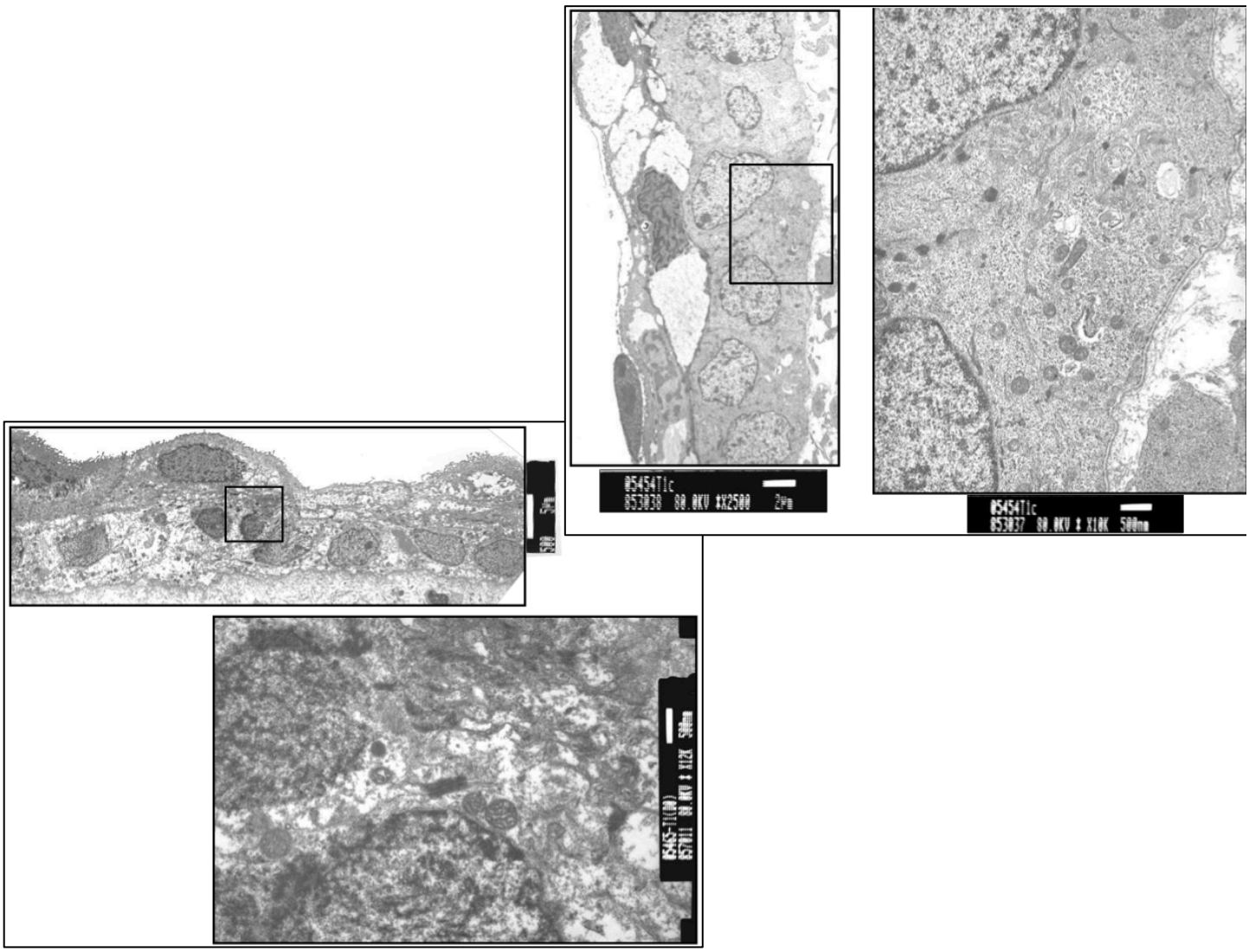
A. Caucasian, 21 years old, 160 cm x 55 kg, first pregnancy, 11+2 weeks of gestation, negative anamnesis;
B. Caucasian, 24 years old, 155 cm x 48 kg, second pregnancy (first miscarriage), 12 weeks of gestation, negative anamnesis;
C. Caucasian, 23 years old, 155 cm x 52 kg, first pregnancy, 11 weeks of gestation, negative anamnesis;
D. Caucasian, 21 years old, 169 cm x 53 kg, first pregnancy, 12+3 weeks of gestation, negative anamnesis.

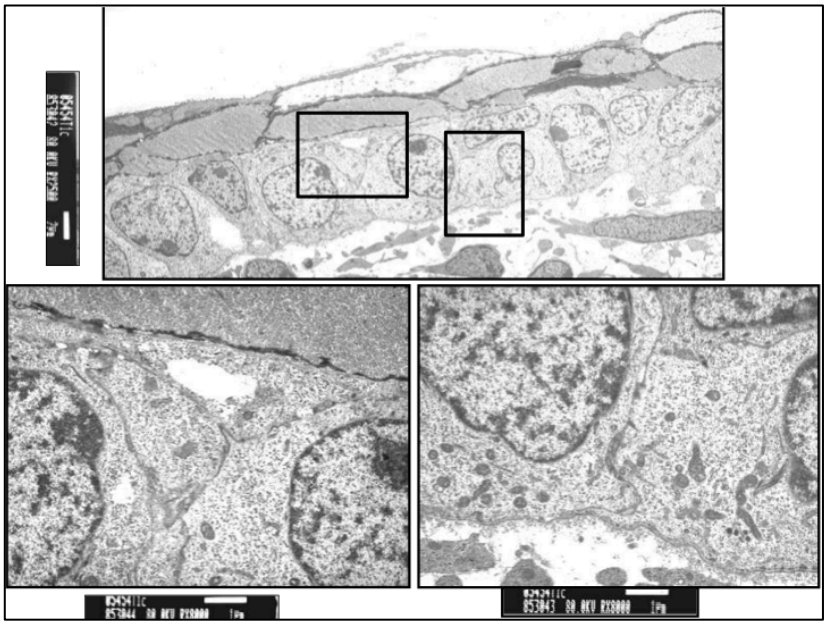
Each sample were performed and analyzed in accordance with the protocol developed and previously discussed, for a total of 72 samples.

The results of skin and muscle TEM analysis will be shown in chronological order from T1 to T9.

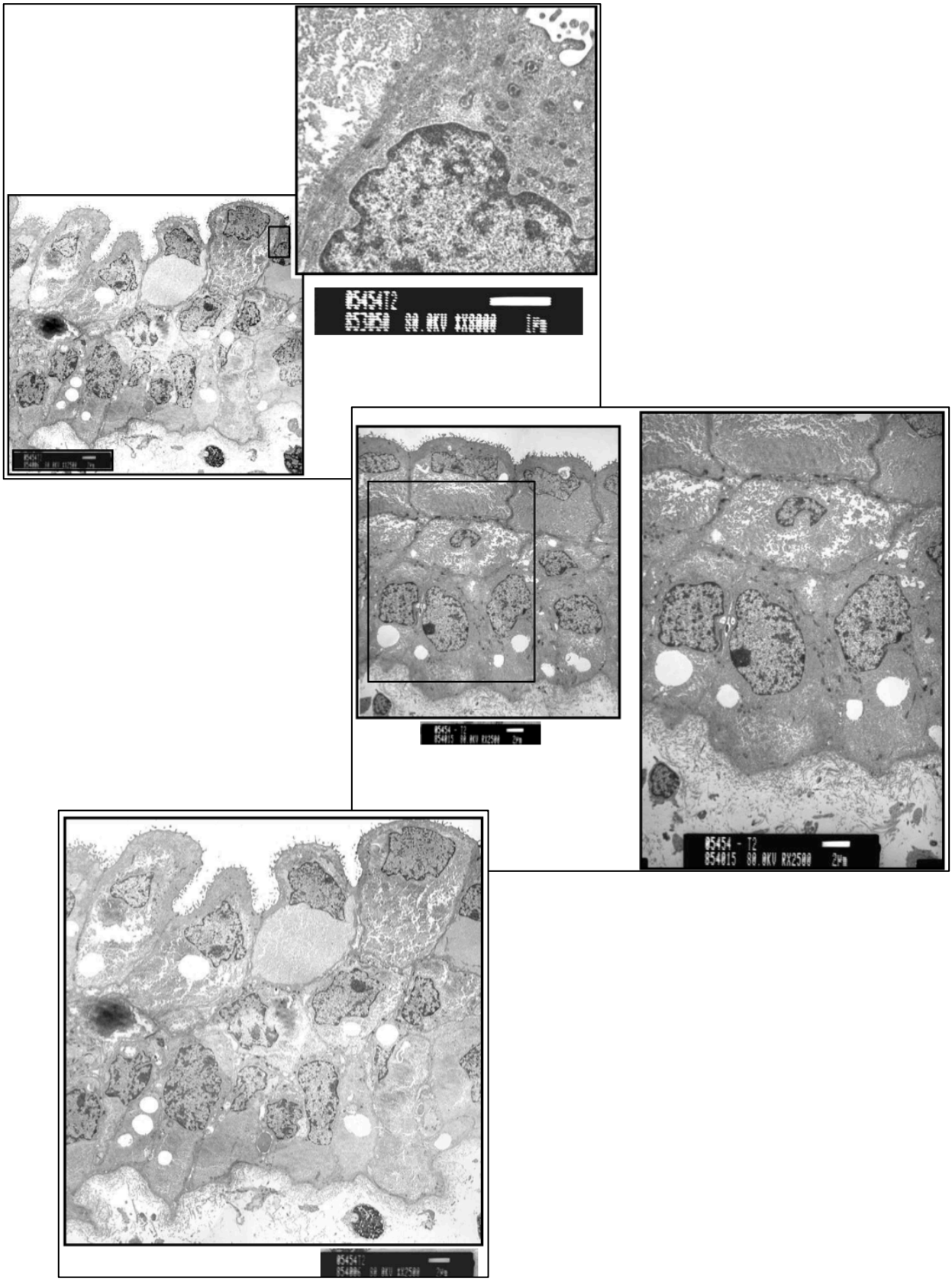
The results of optical microscope analysis were all negative: no alteration was observed in the different sampling times for both skin and muscle.

SKIN TEM ANALYSIS: T1 (0 MIN.)

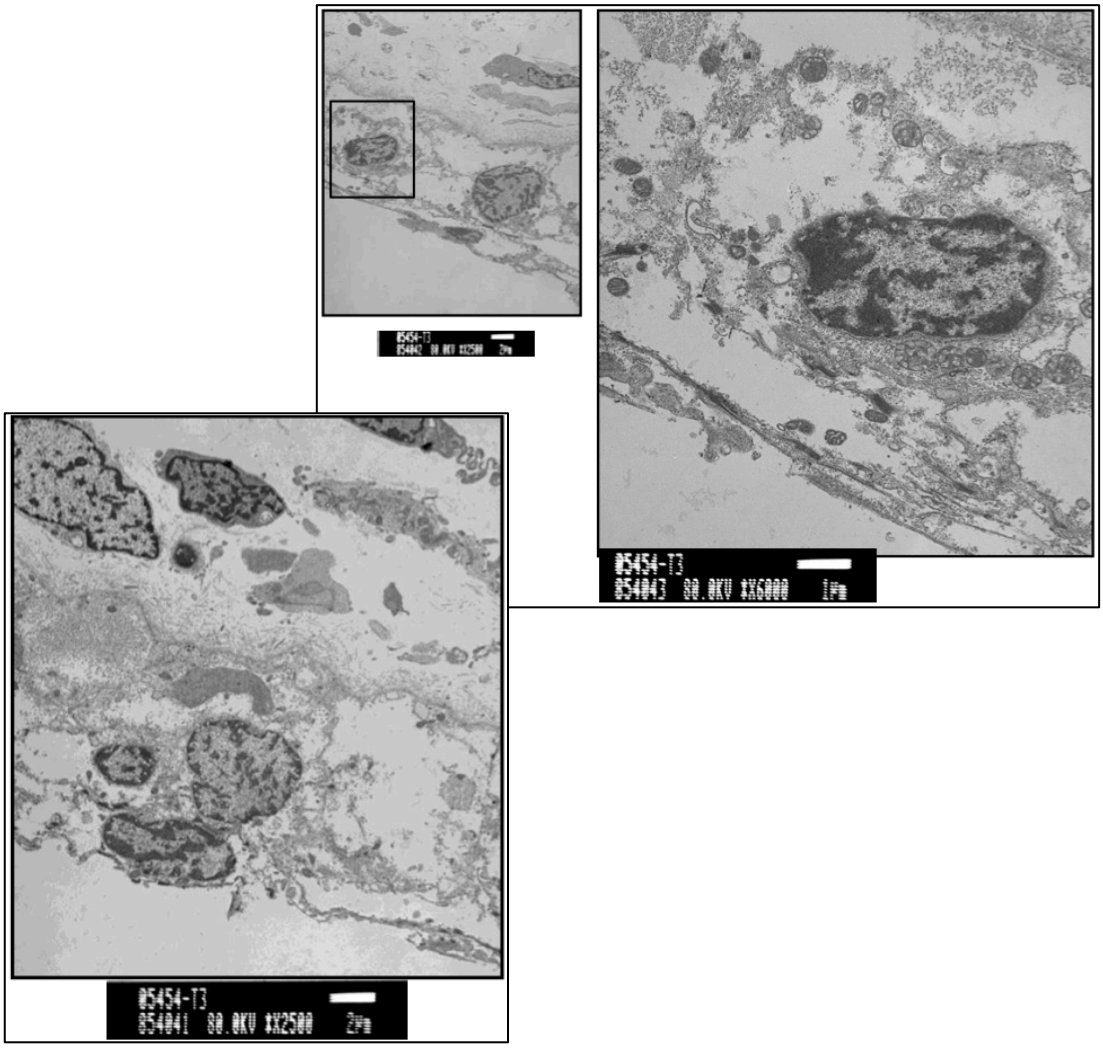


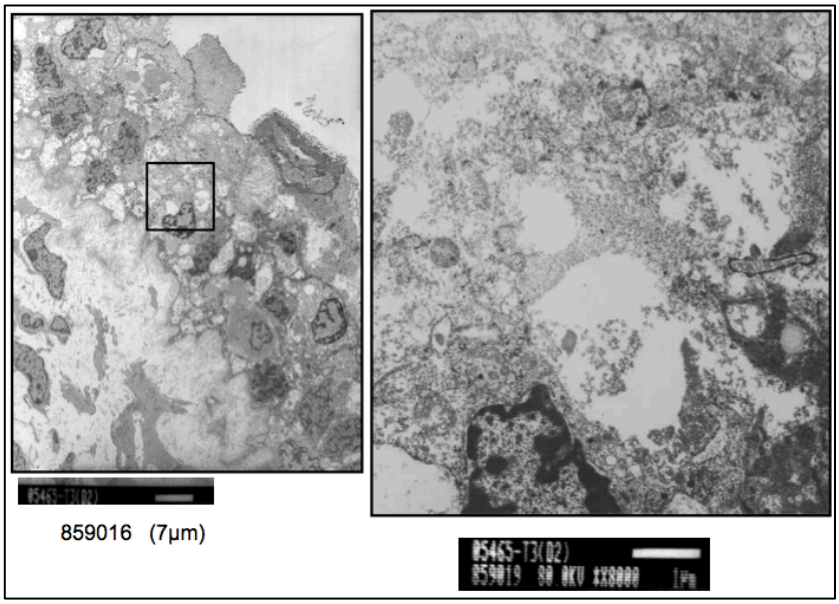


SKIN TEM ANALYSIS: T2 (15 MIN.)

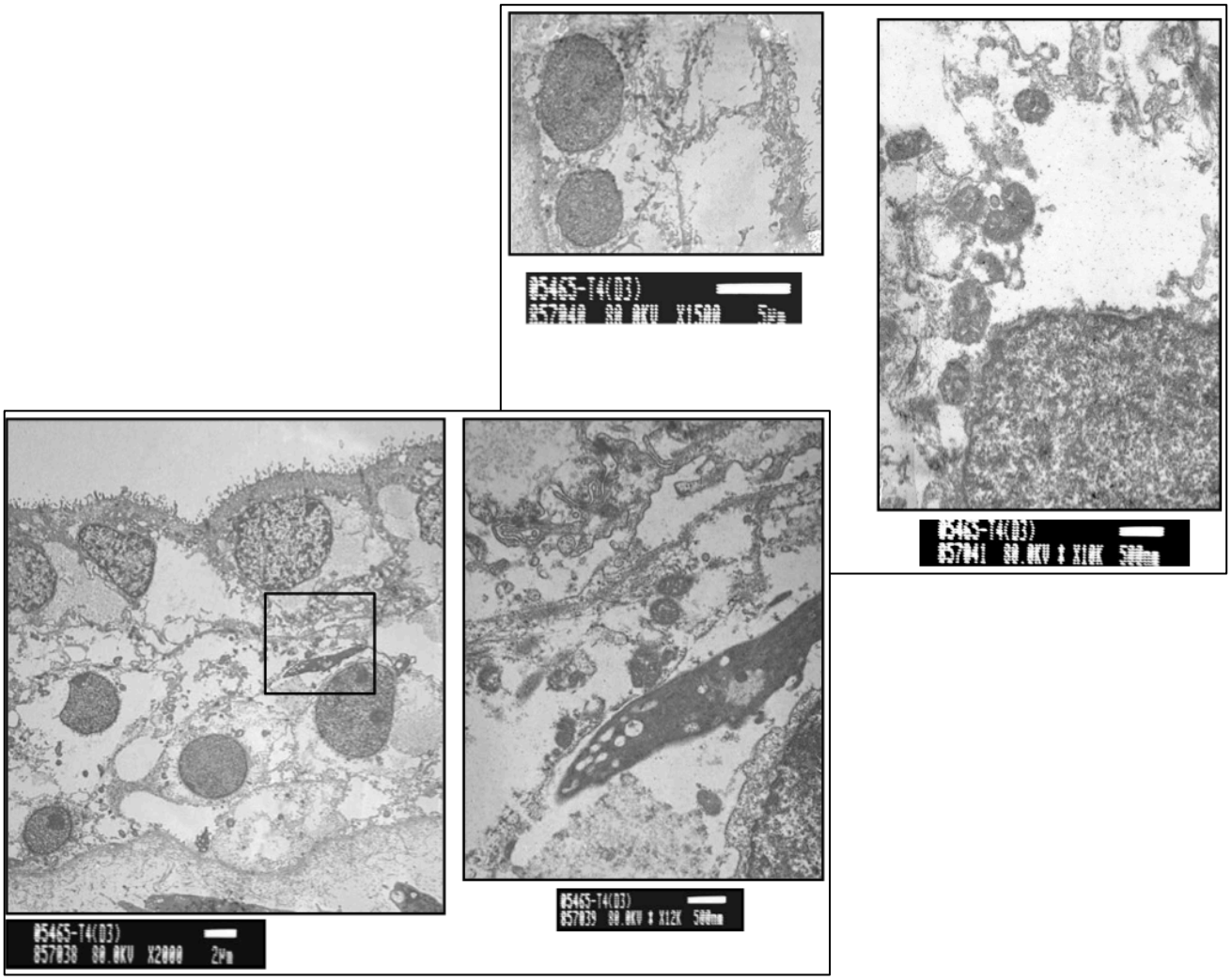


SKIN TEM ANALYSIS: T3 (30 MIN.)

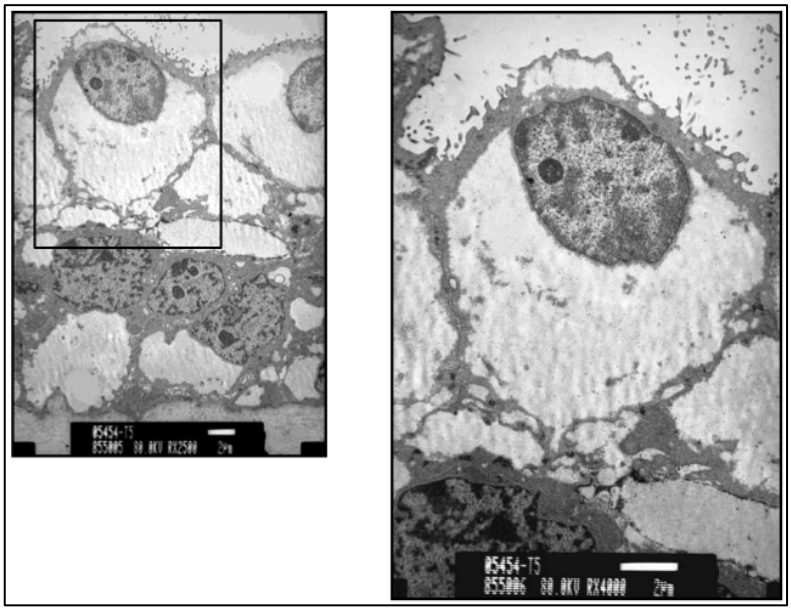


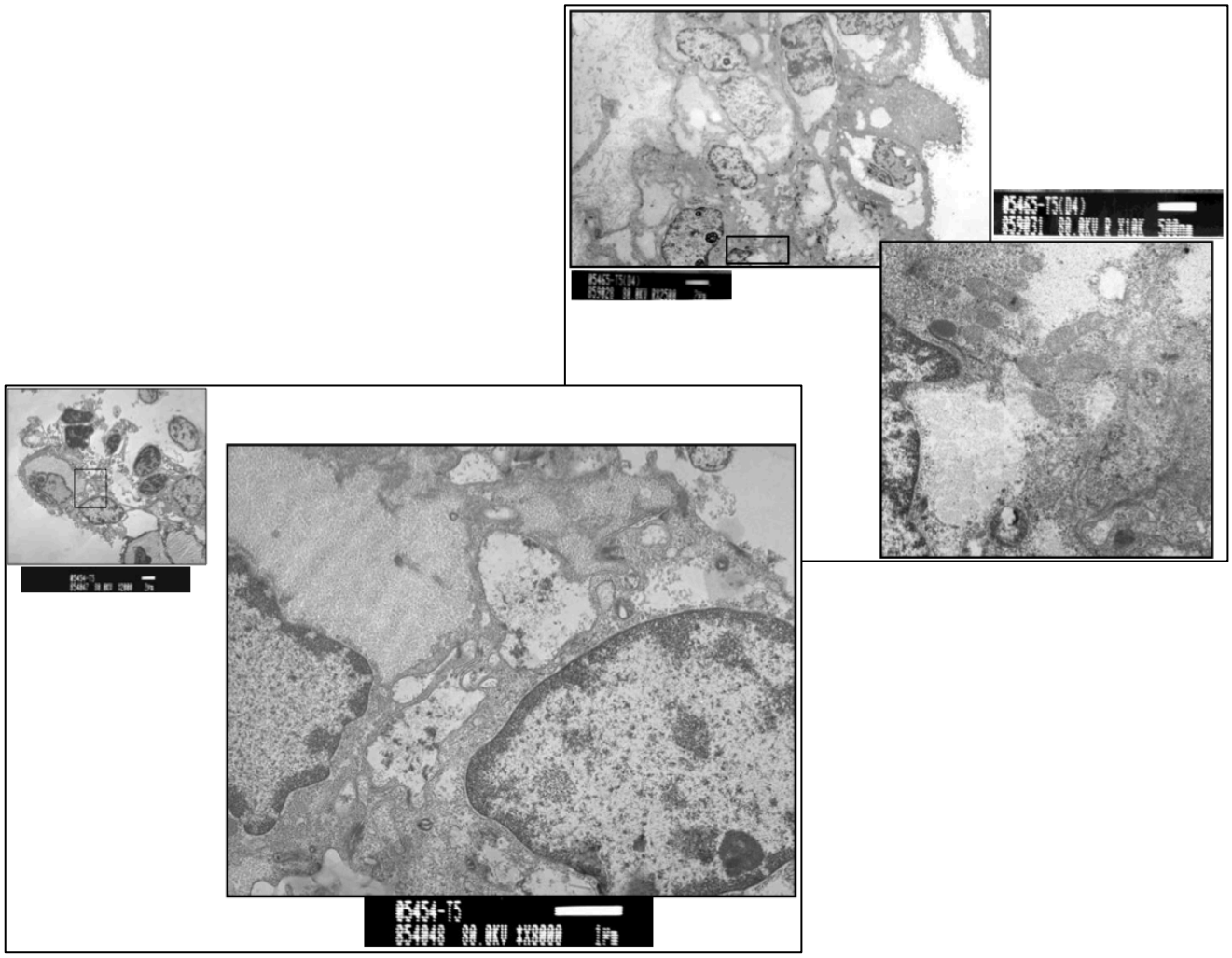


SKIN TEM ANALYSIS: T4 (60 MIN.)

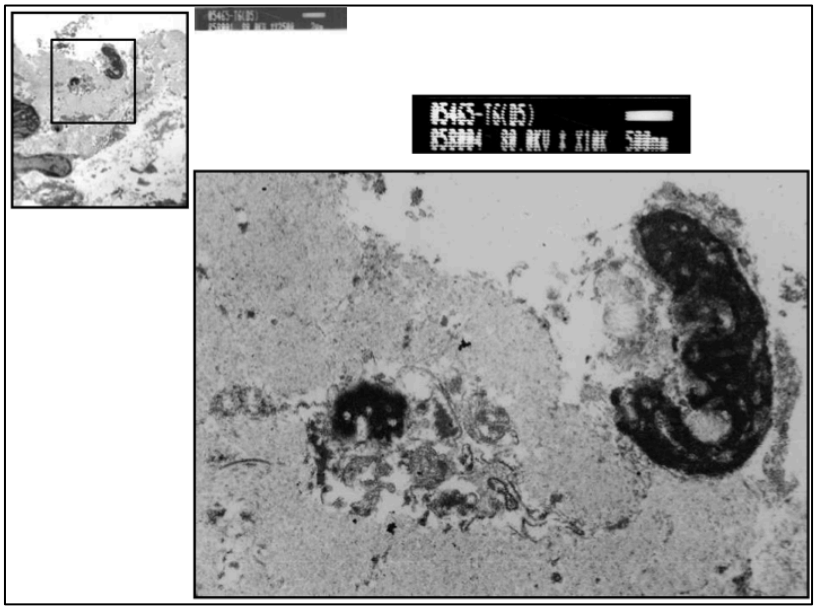


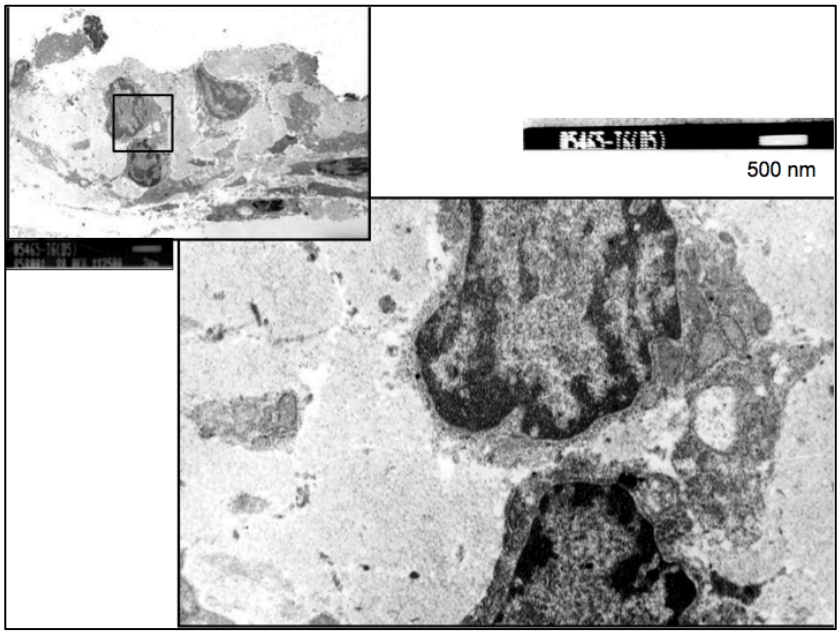
SKIN TEM ANALYSIS: T5 (90 MIN.)



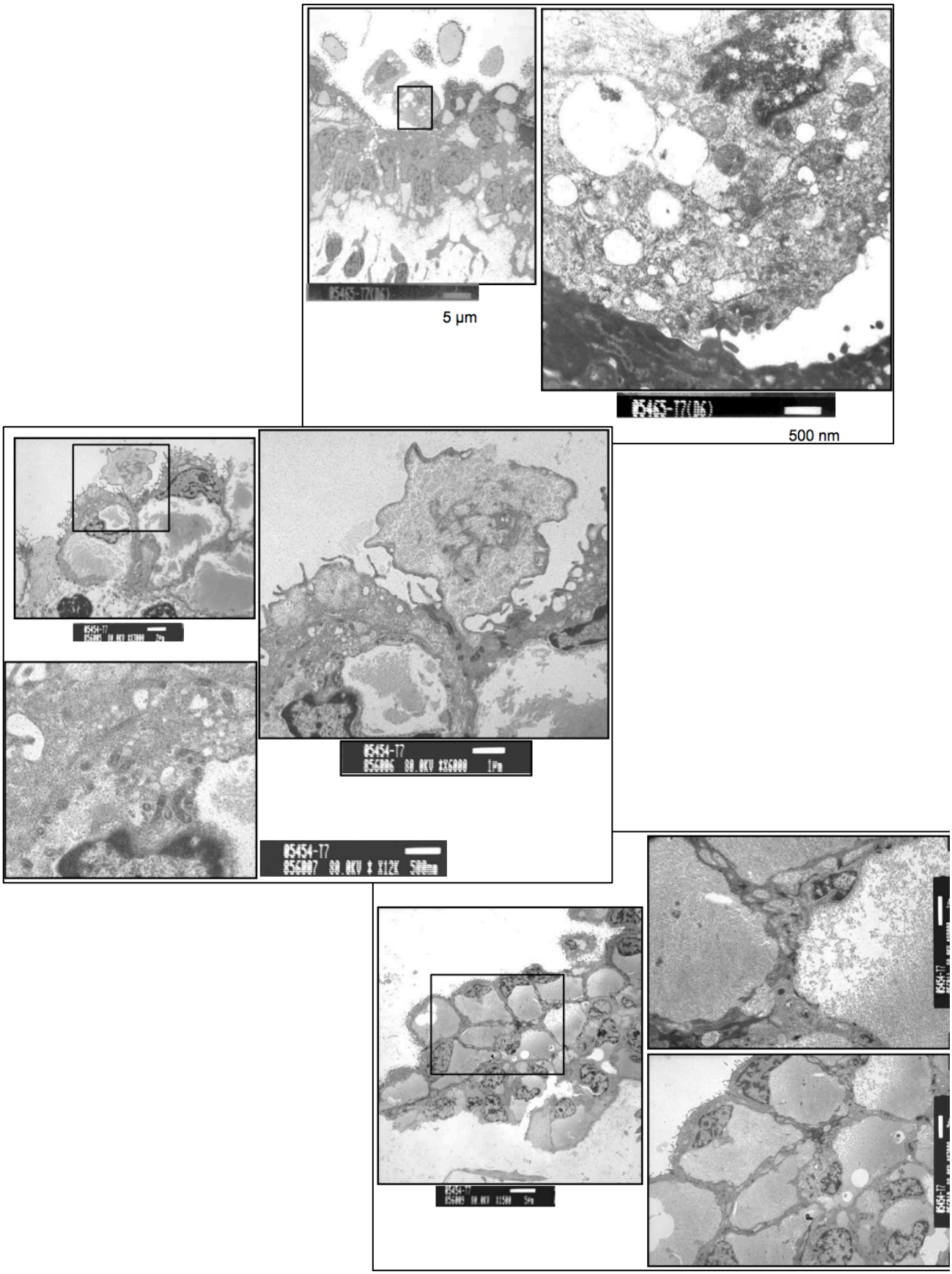


SKIN TEM ANALYSIS: T6 (2 H.)

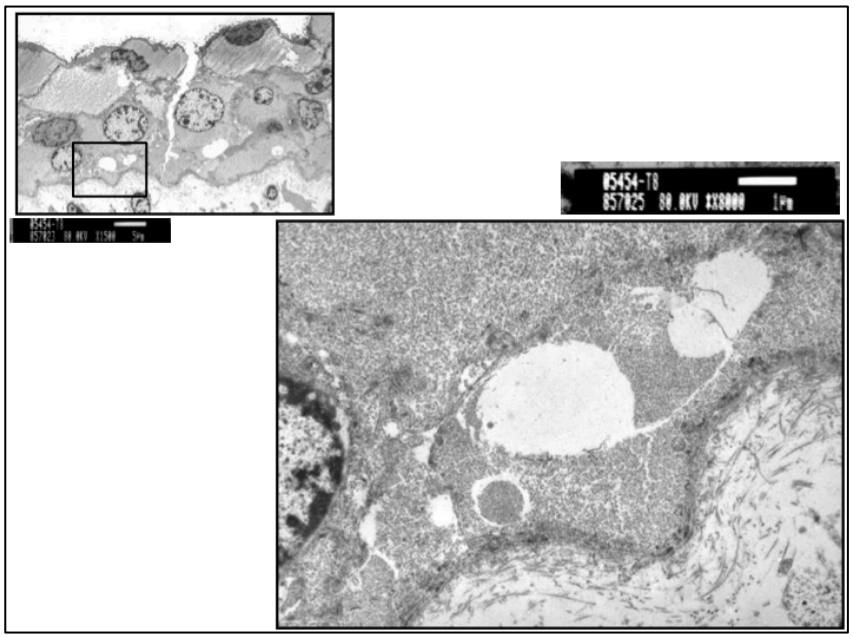


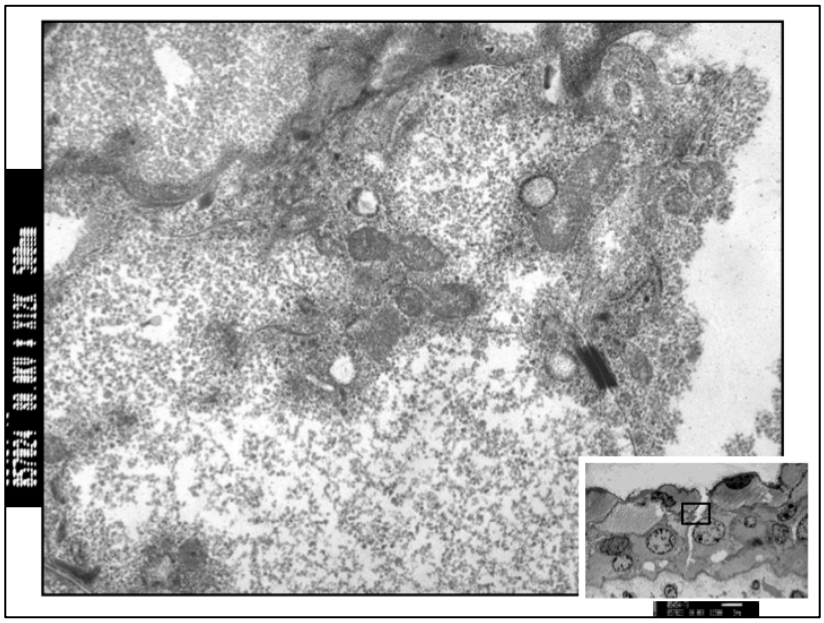


SKIN TEM ANALYSIS: T7 (3 H.)

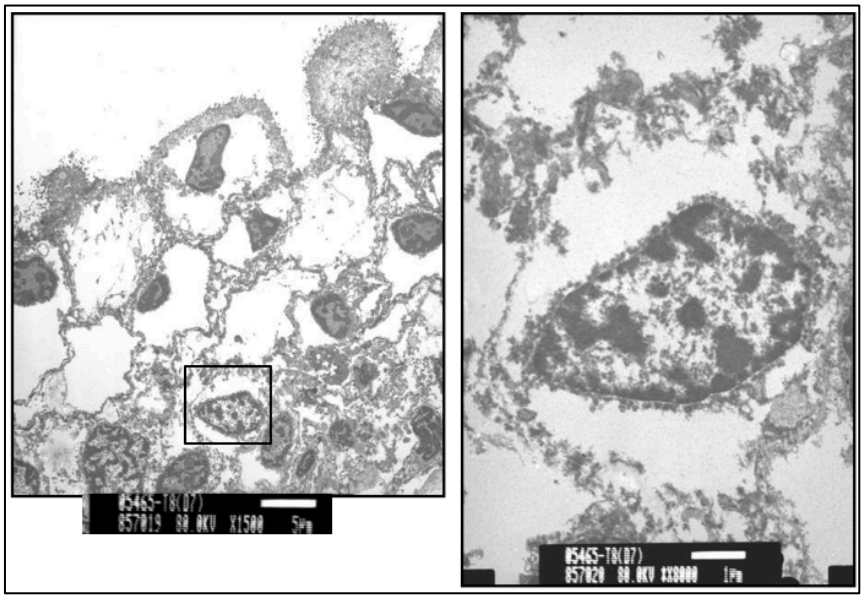


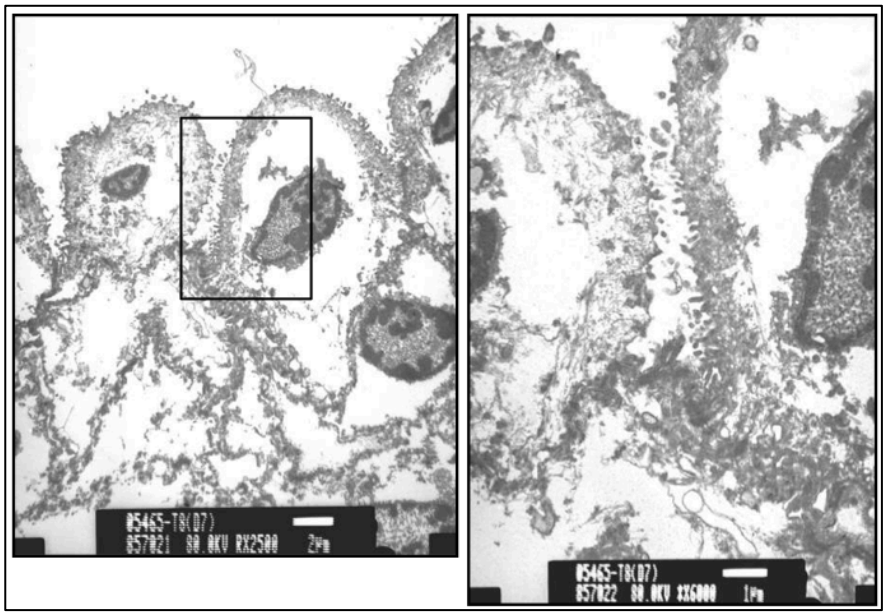
SKIN TEM ANALYSIS: T8 (4 H.)



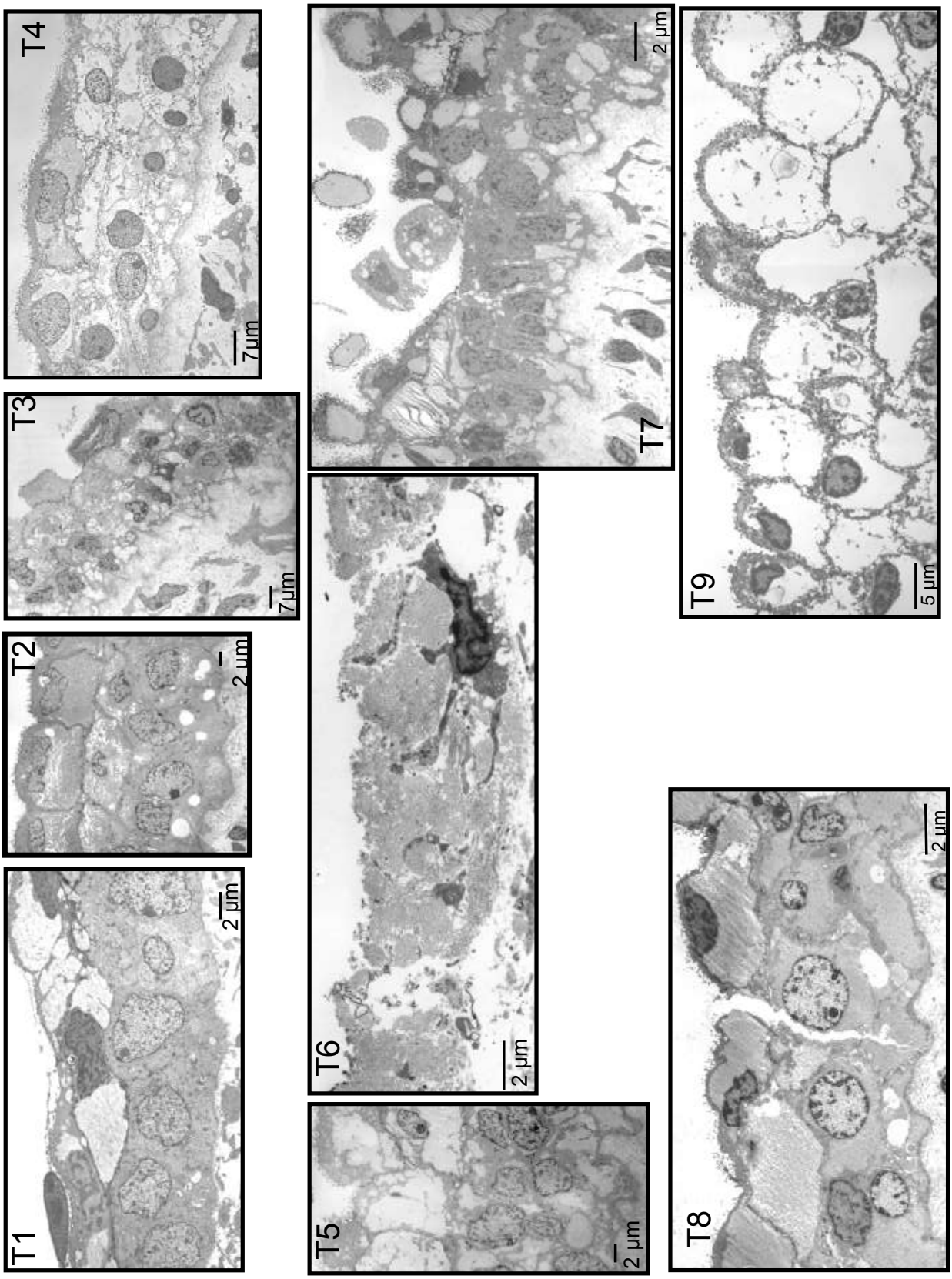


SKIN TEM ANALYSIS: T9 (20 H.)

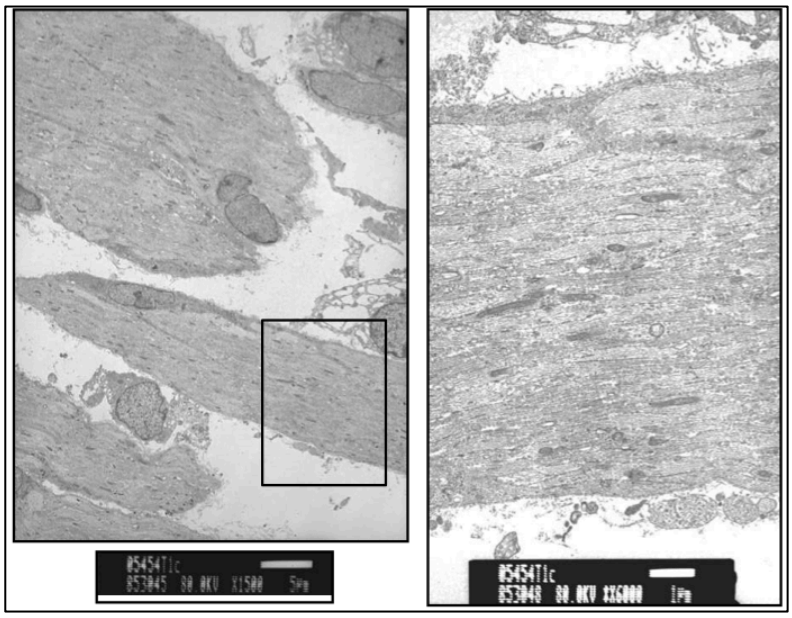


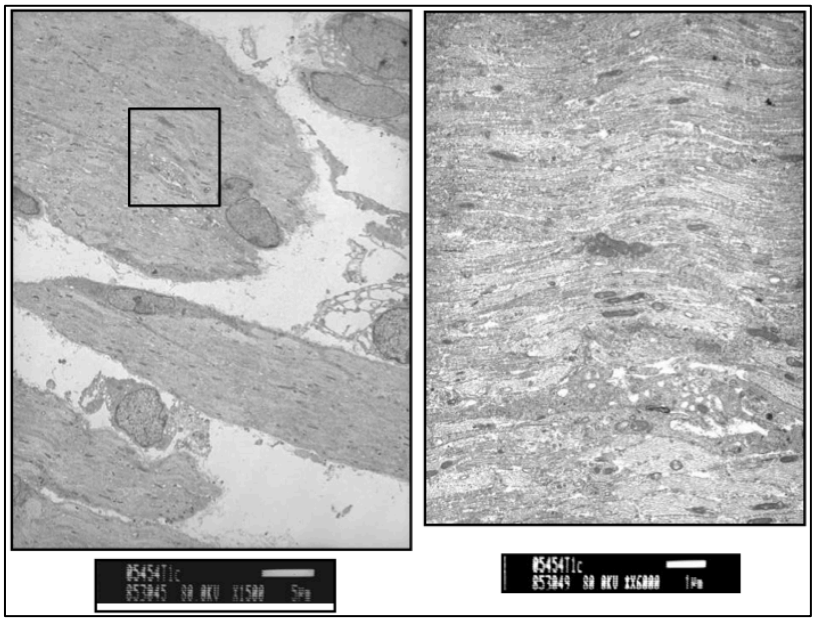


SKIN TEM ANALYSIS: SUMMARY TABLE

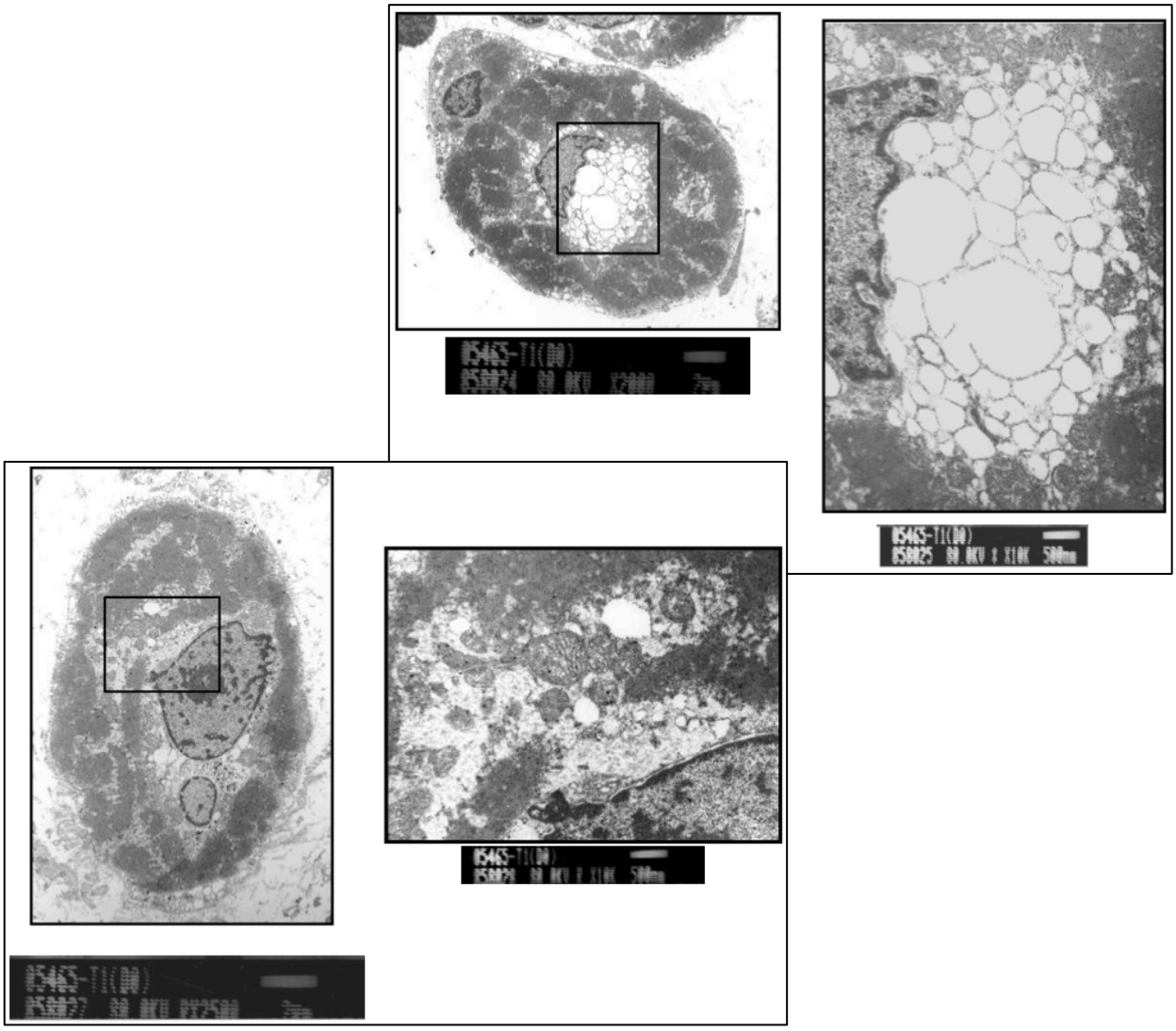


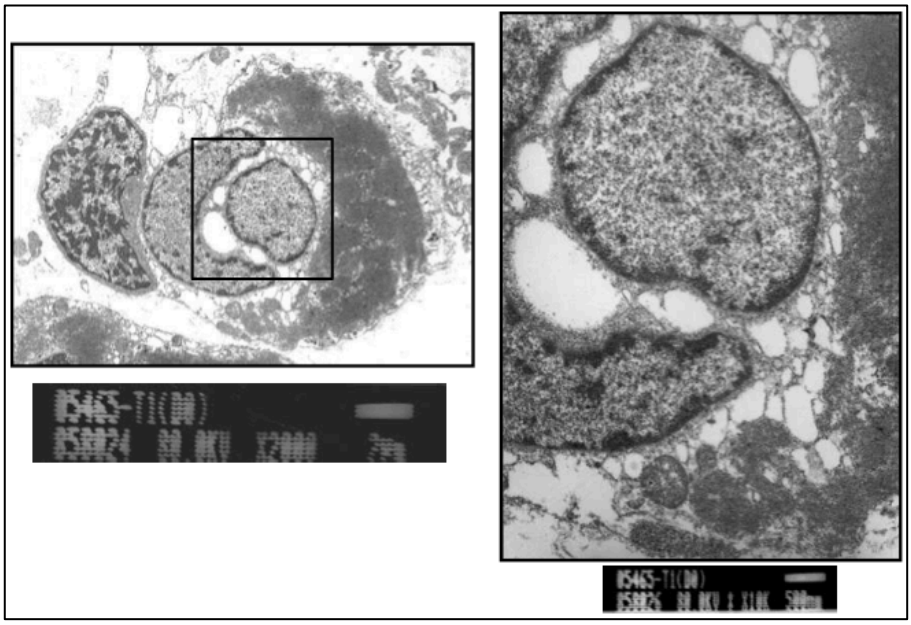
MUSCLE TEM ANALYSIS: T1 (0 MIN.)



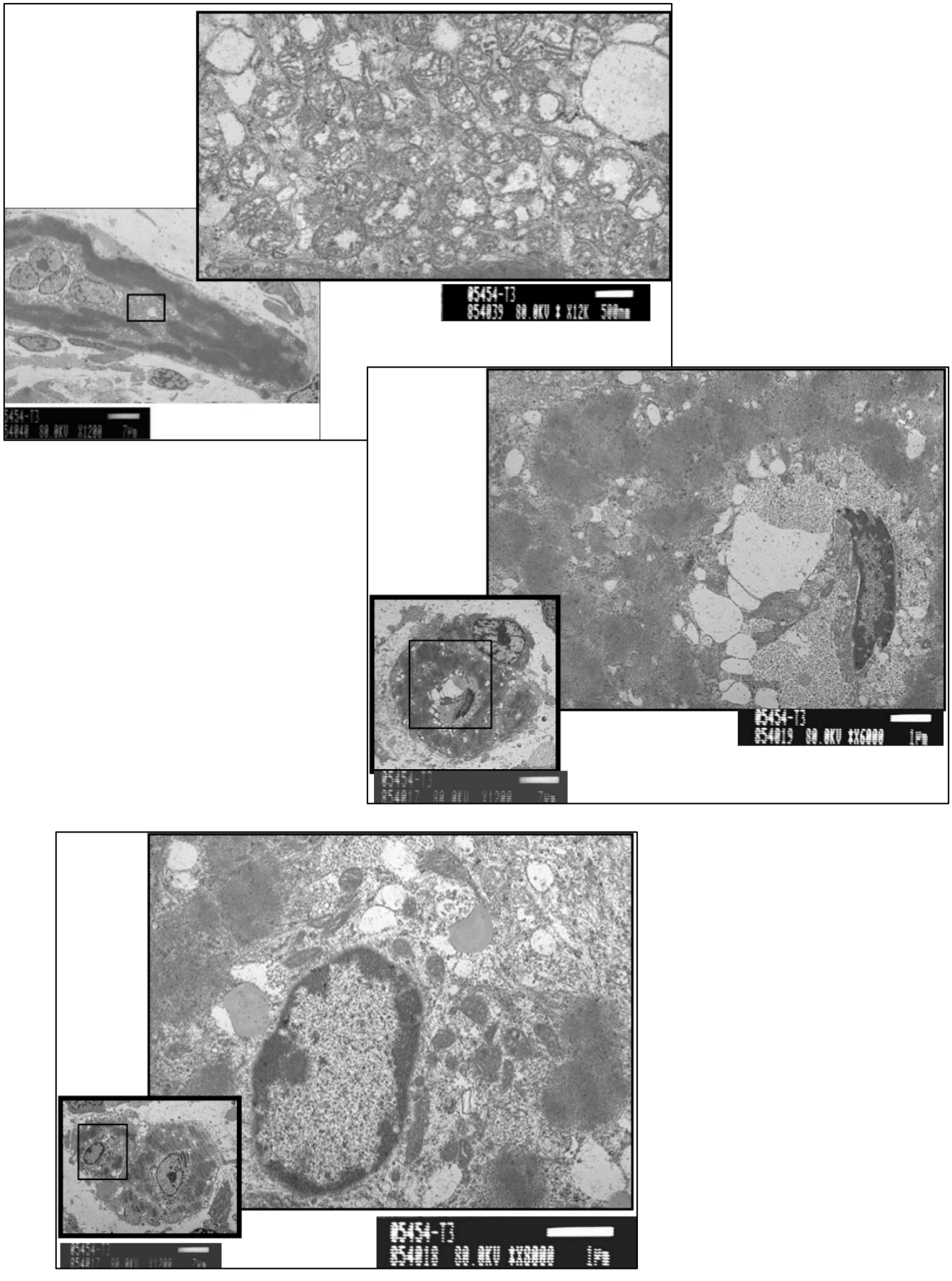


MUSCLE TEM ANALYSIS: T2 (15 MIN.)

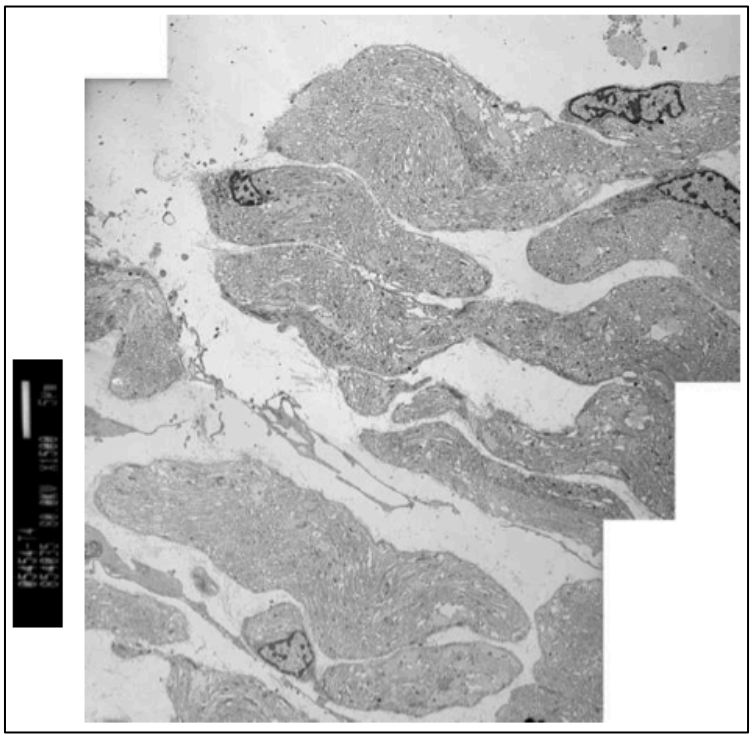


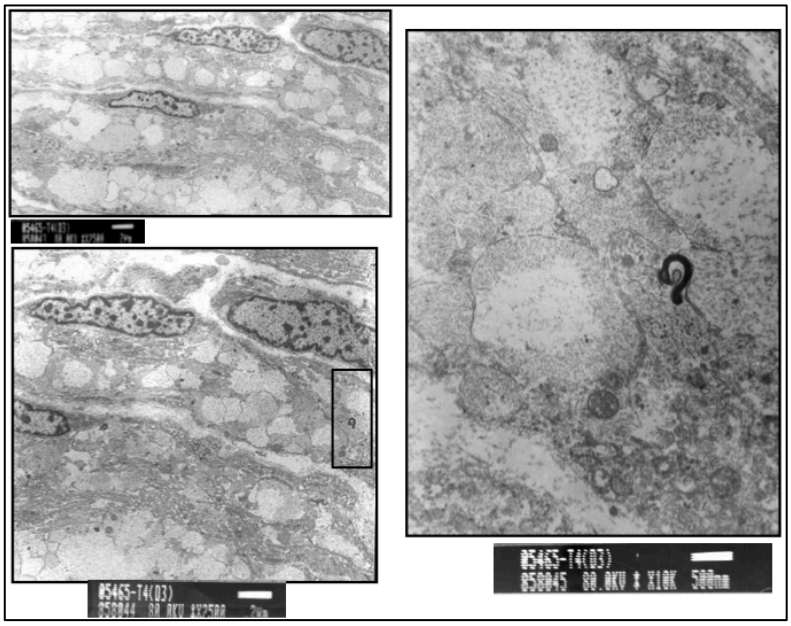


MUSCLE TEM ANALYSIS: T3 (30 MIN.)

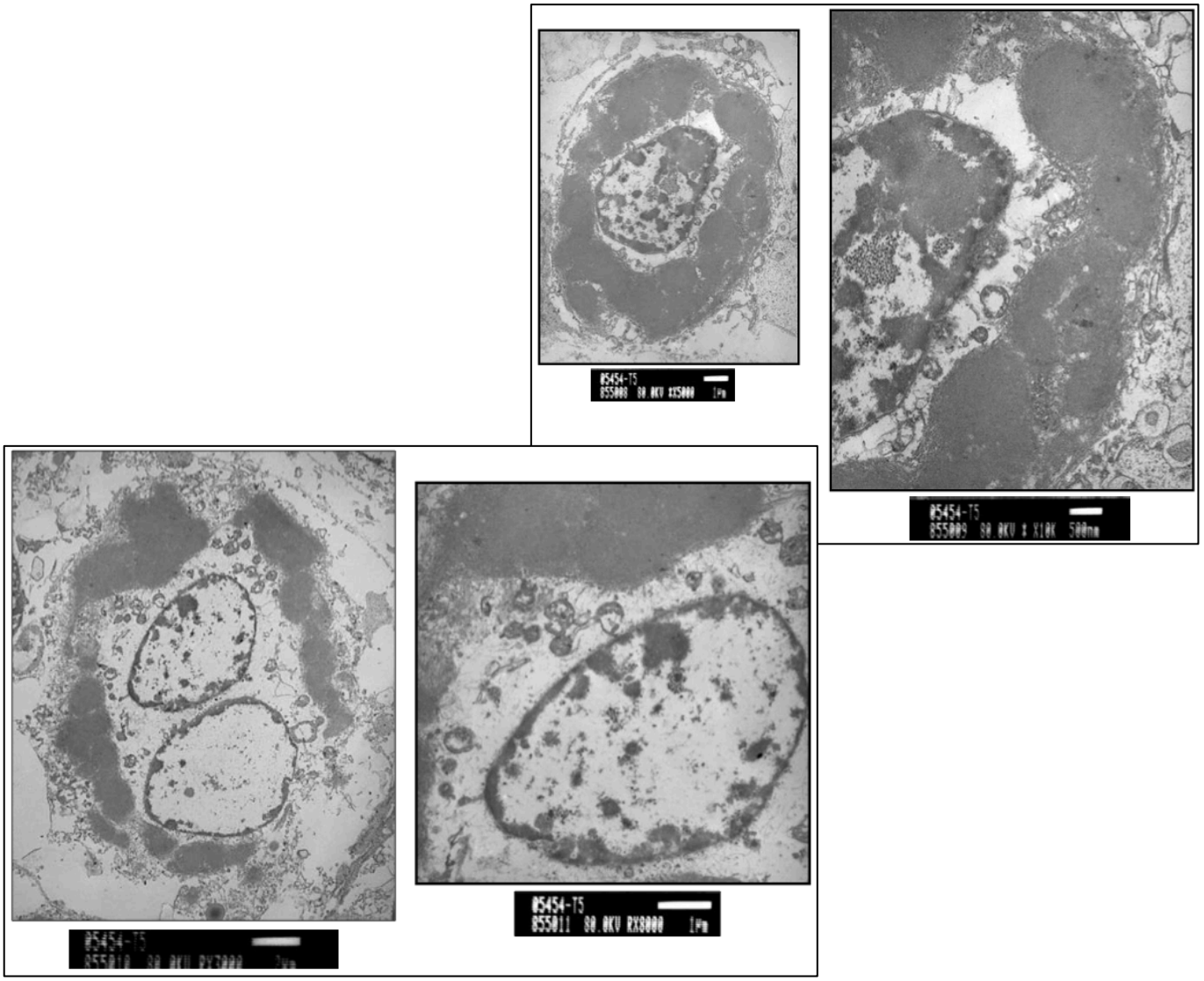


MUSCLE TEM ANALYSIS: T4 (60 MIN.)

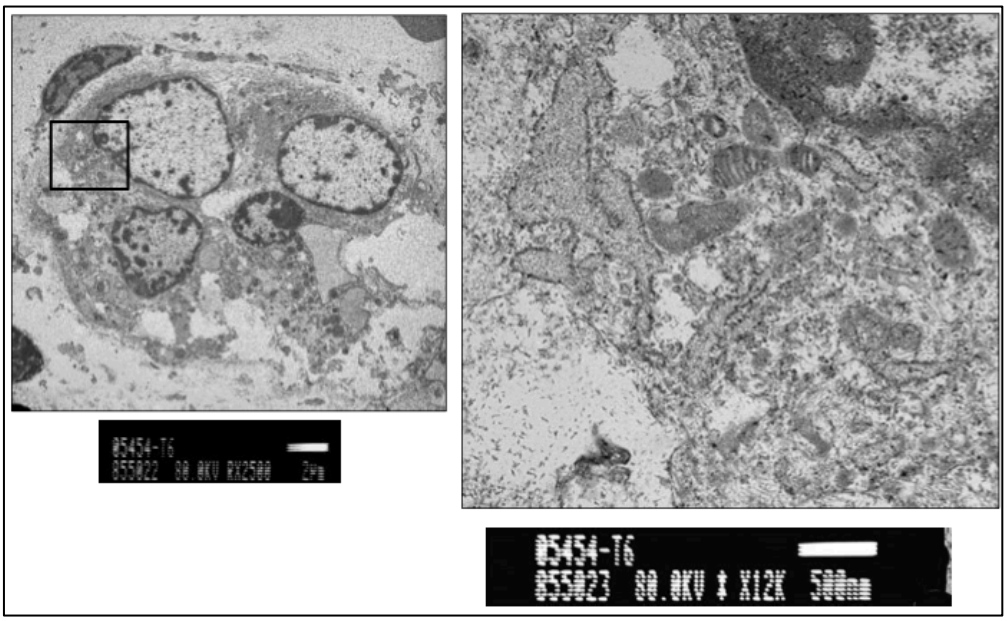


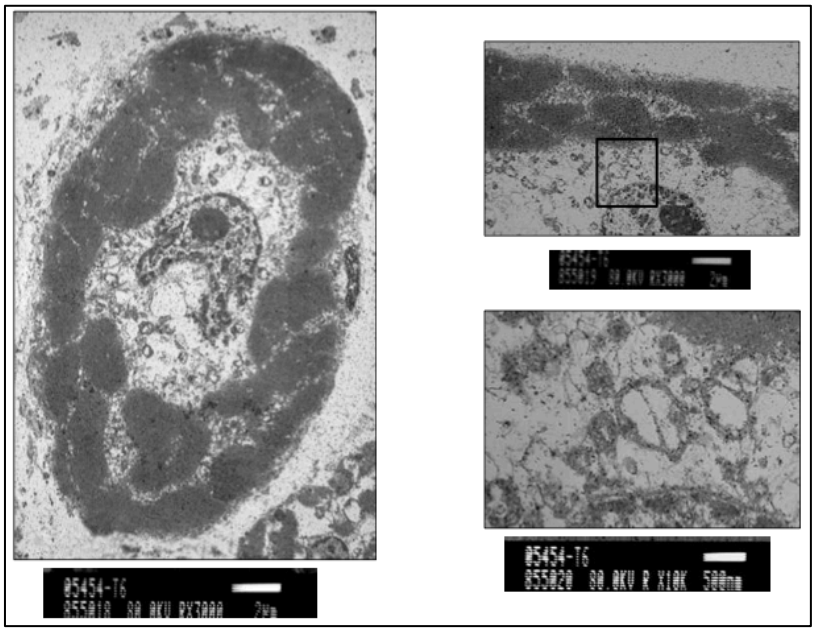


MUSCLE TEM ANALYSIS: T5 (90 MIN.)

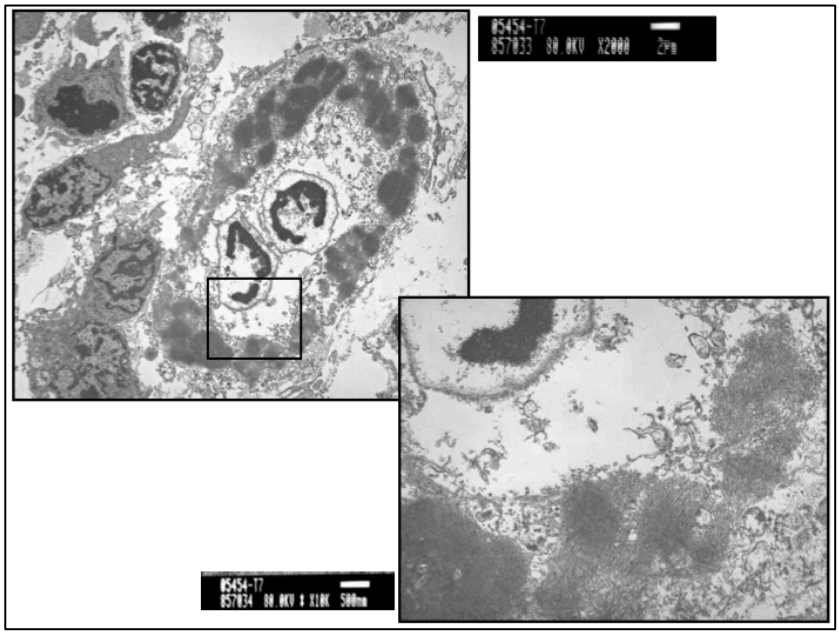


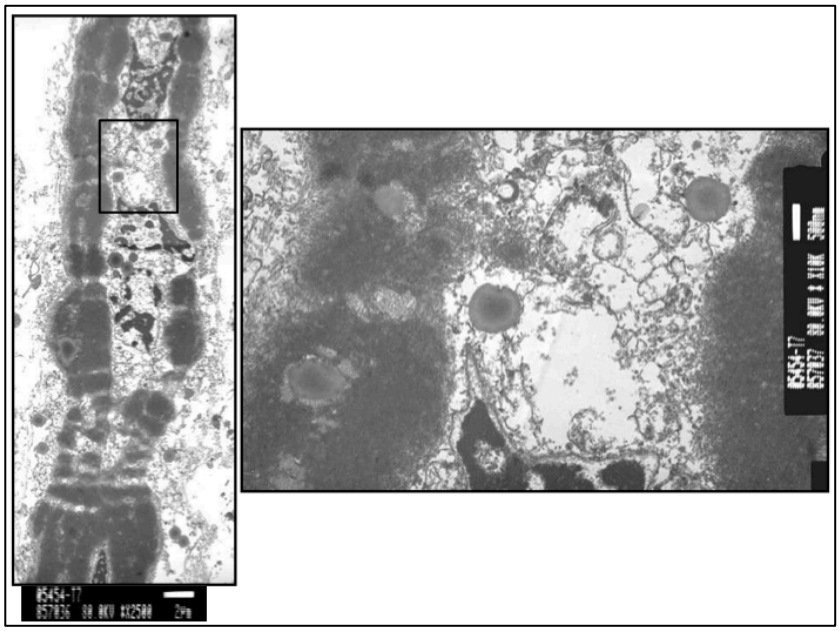
MUSCLE TEM ANALYSIS: T6 (2 H.)





MUSCLE TEM ANALYSIS: T7 (3 H.)





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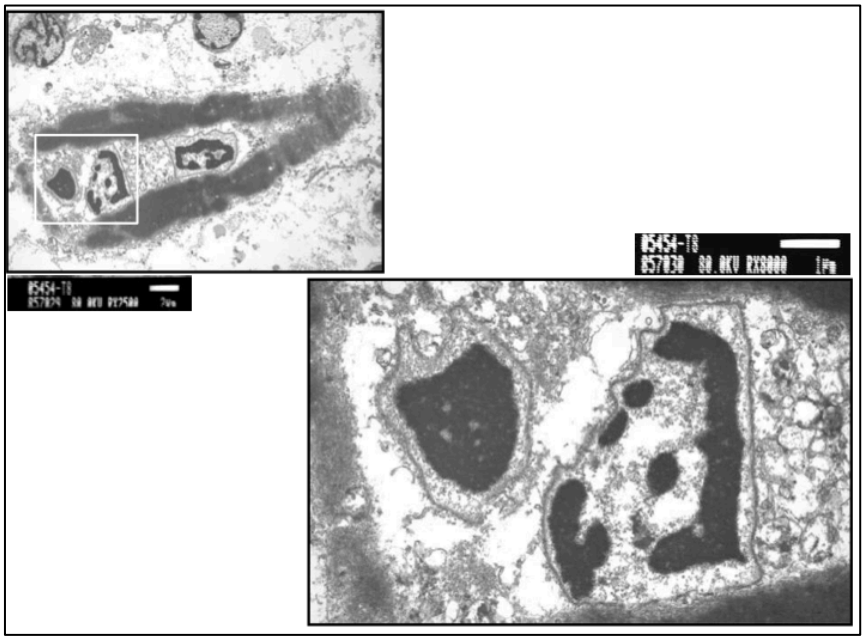

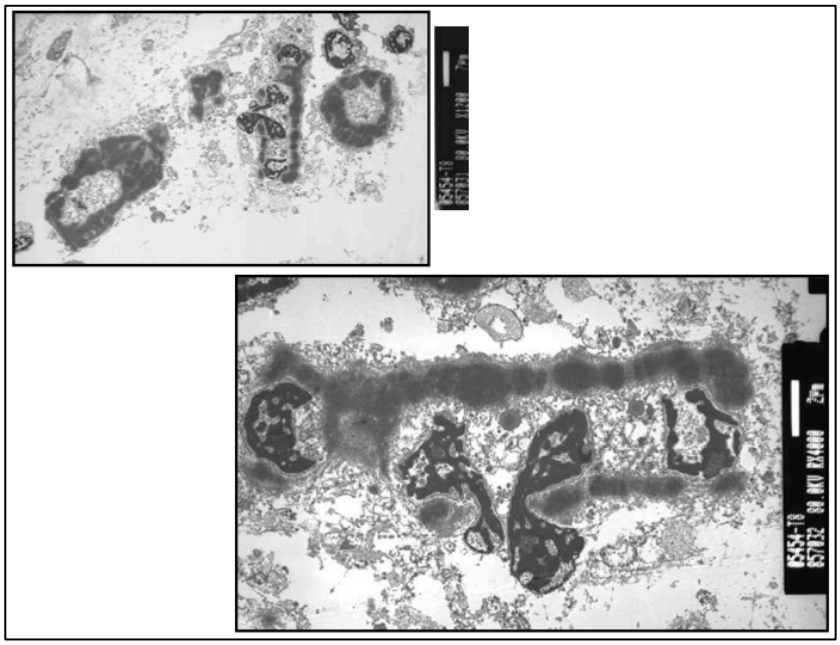

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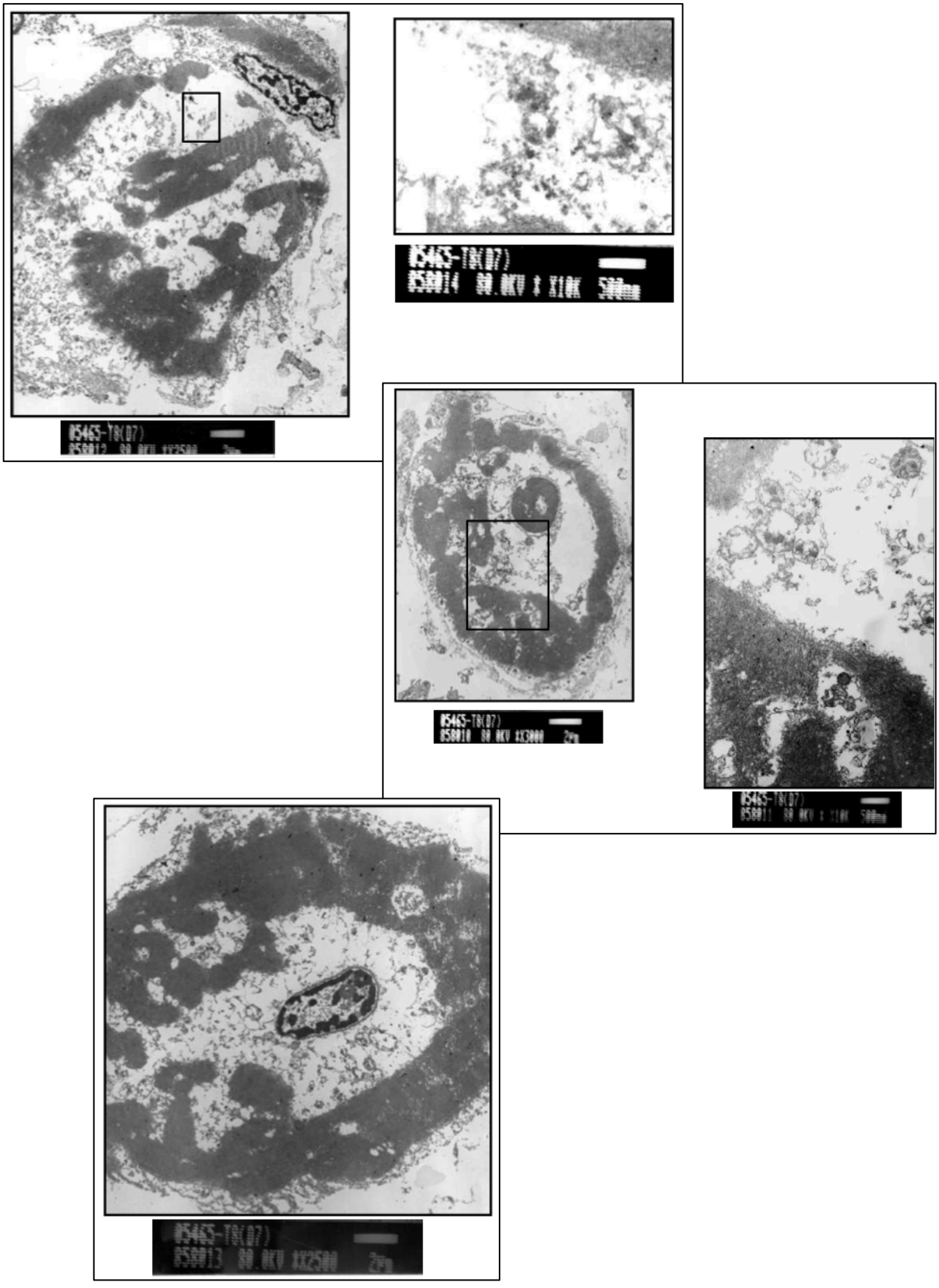
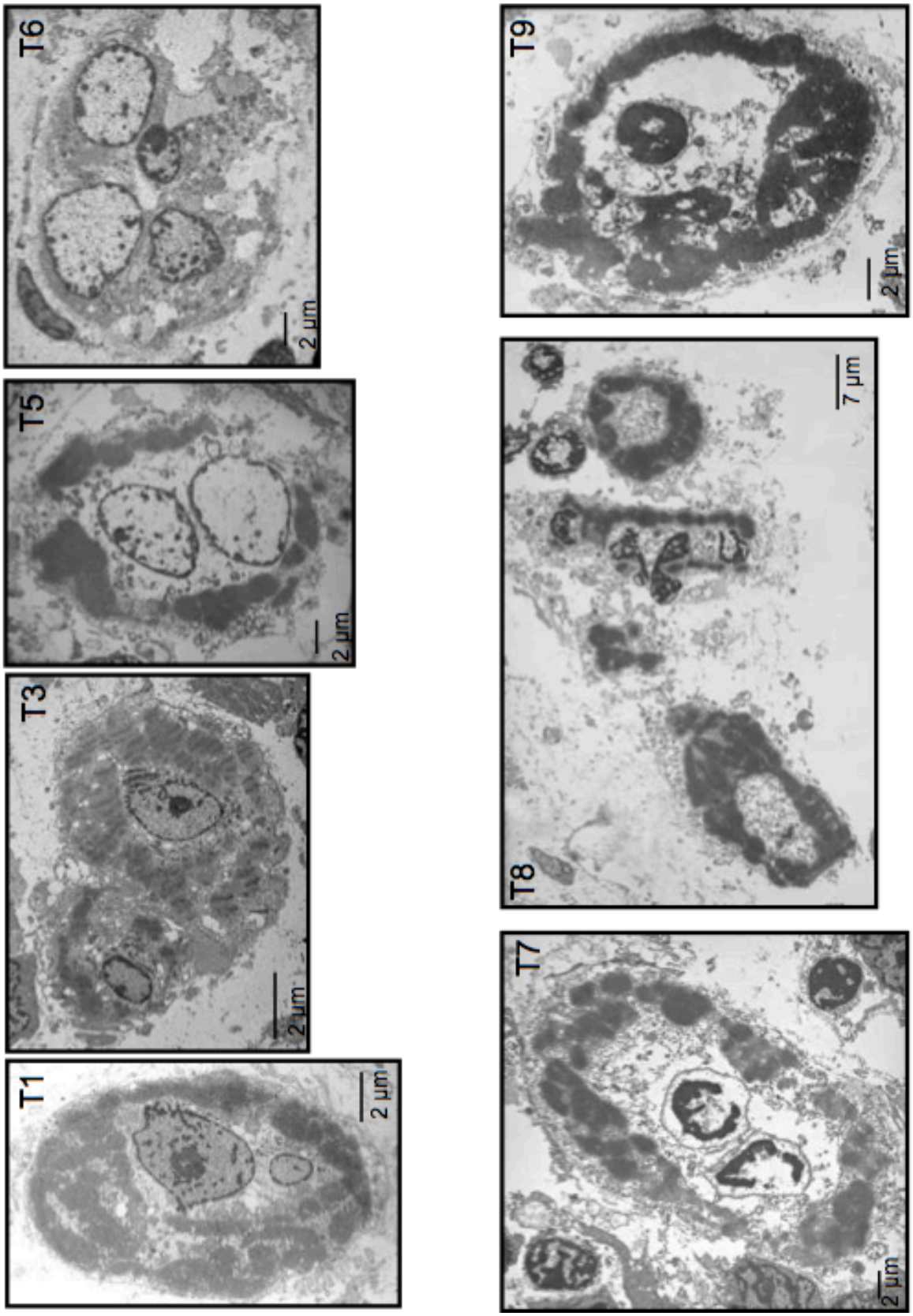
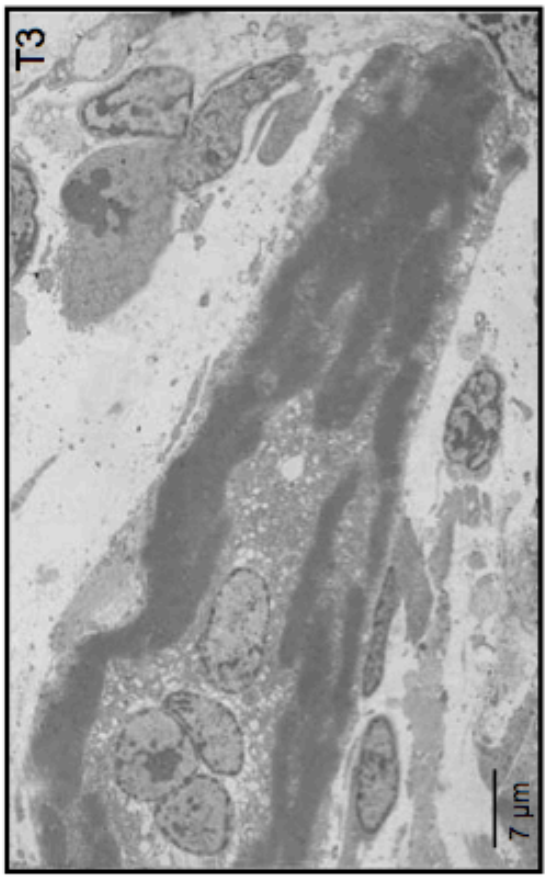
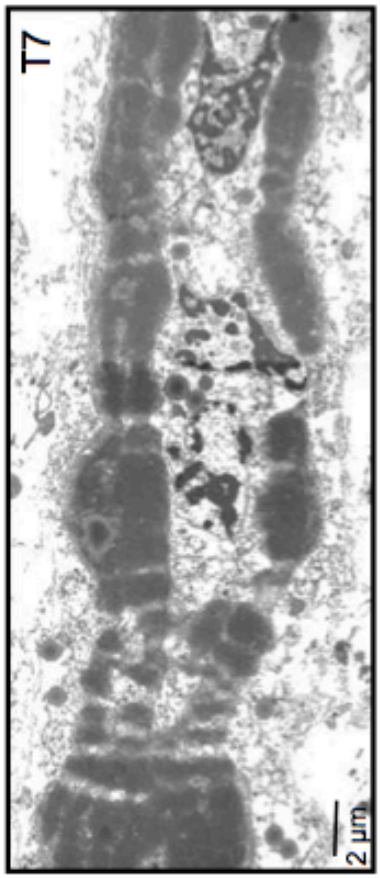


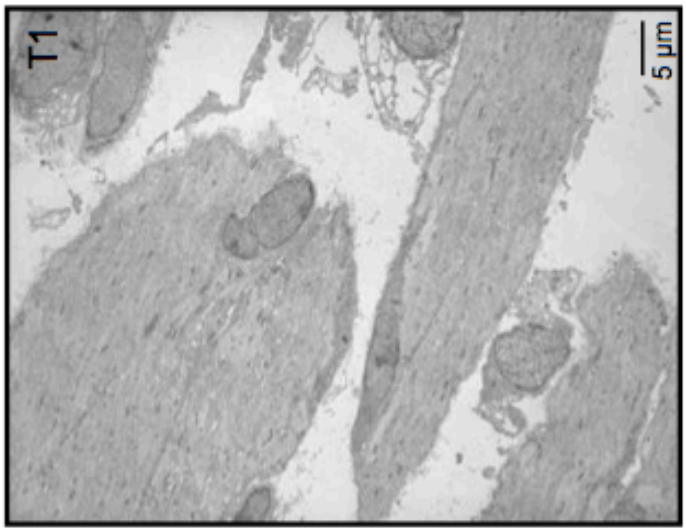
MUSCLE TEM ANALYSIS: SUMMARY TABLE

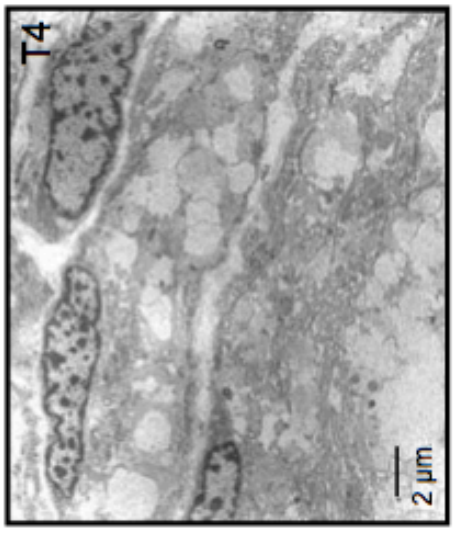


MUSCLE TEM ANALYSIS: SUMMARY TABLE









4E. DISCUSSION

The characteristics of the study population, however limited to four cases, were found to be fully comparable in terms of age (21-24 years), week of pregnancy (11-12) and, especially, for the absence of maternal and fetal diseases.

All cases were subjected to the experimental protocol with the results that have been iconographically exposed in the previous paragraph.

The main element emerged from the TEM analysis positively correlated with the time of death has been a steady and substantial increase in tissue vacuolization, both in skin and in muscle, although in the latter to a lesser extent. These vacuoles are well defined within the surrounding cellular structures and are distinguishable in quali-quantitative terms. It is not possible that such a vacuolization can be traced to defects in the fitting or preparation of the samples, since cellular membranes and all sub-cellular structures are still intact and legible. As it is possible to observe in the results tables, this progressive vacuolization affects every area of the cell and does not have a characteristic distribution or restricted to certain elements: this characteristic of "irregular diffusion" of the phenomenon is common to all the thanatological events also in the adult body.

The vacuolization is observed from the first observation time (T2, 15 minutes) and manifests itself qualitatively greater with increasing time of death. In general, as mentioned, the muscular structures are more resistant than those of the skin, manifesting such phenomena with a slower progression and a more limited diffusion (in agreement with the literature of ultrastructural morphology - see, in this regard, the study of Tomita et al. 2004).

Another matter of interest, observing other sub-cellular structures, is the conservation status of the mitochondria. These organelles show alterations always ranging from mild to high, but entirely independent from the time of death. The data is, therefore, inconstant and probably related to other variables (for example, pH): for this reason it does not seem possible its use in the chronology of fetal death.

Finally, it is worth mentioning the state of perfect preservation of cell junctions, even at maximum time after death.

As regards to the analysis of the same preparations at optical microscope, it is recalled, this has not allowed us to observe any alteration within the time analyzed.

Excluding the phenomenon of vacuolization, the results are entirely consistent with the literature reviewed, which showed no microscopic alteration within the first 24 hours in skin or skeletal muscle.

In order to try to quantify the extent of vacuolization, semi-fine sections of skin samples were observed by optical microscope and were performed the count of the number of vacuoles in different fields.

In particular, our four study cases were analyzed at three different time points (T1, T3, T8). The counting was performed by photographing three fields for each section with 40x objective and counted all the vacuoles (Images 1-3: vacuoles can be observed as optically empty white circles). Then was later made the arithmetic mean obtaining a value for each time point. The software used for cell counting is UTHSCSA Image Tool 3.0.

It was observed in this way a significant increase in the average number of vacuoles from T1 to T8: in particular, at T1 (pick-up time) were counted an average of 46 vacuoles on three fields, while the medium number of vacuoles to T8 (4 hours) was 123 (total increase of 167.4%). An intermediate value has been counted at T3 (30 minutes), where the average number of vacuoles was 77 (+ 67.4% compared to T1).

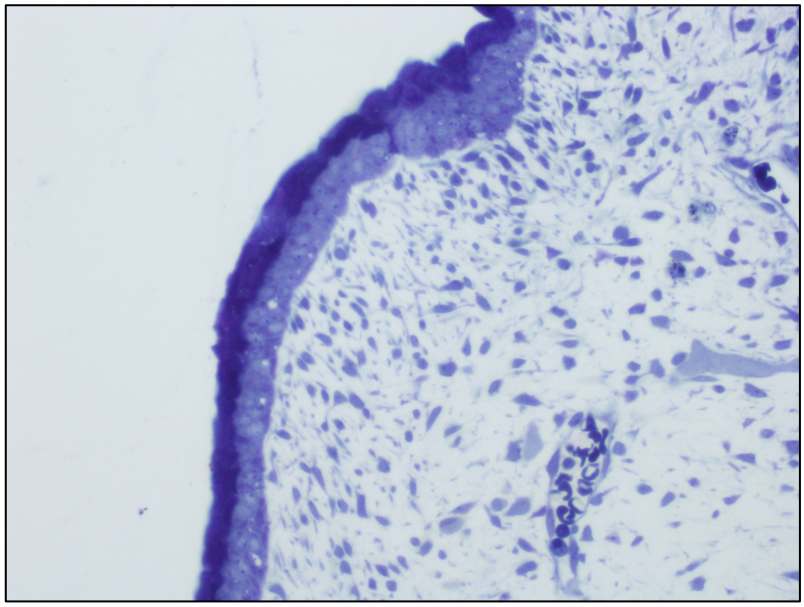


Image 1. *Semi-fine section of skin at T1, 40x.*

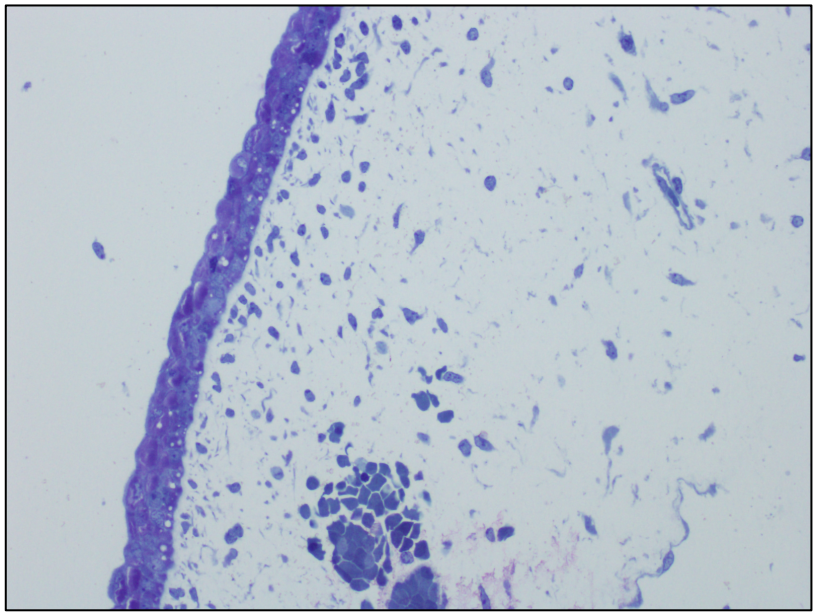


Image 2. *Semi-fine section of skin at T3, 40x.*

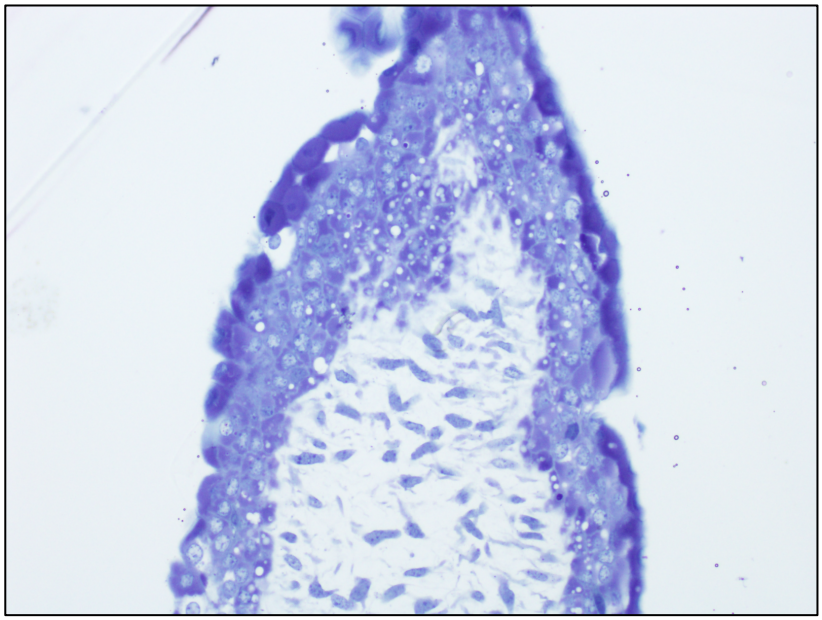


Image 3. *Semi-fine section of skin at T8, 40x.*

These preliminary quantitative observation must now be extended to other time points and a quantification of the areas of vacuoles must be performed.

CONSENSO INFORMATO

PROTOCOLLO DI RICERCA SULLA MORTE ENDOUTERINA FETALE

Gent.ma Signora,

l'Ospedale San Paolo collabora con la Sezione di Medicina Legale dell'Università degli Studi di Milano nell'ambito di un progetto di ricerca finalizzato allo studio delle modificazioni che insorgono nel feto dopo la morte, qualora questo venga ritenuto per diverso tempo nell'utero materno.

Questi eventi (morti endouterine fetali) determinano modificazioni morfologiche ancora poco conosciute, la cui comprensione può aiutare i medici che si occupano dello studio della patologia fetale (medici legali, anatomopatologi, ginecologi) a determinarne l'epoca di accadimento e stimare quindi il periodo di tempo in cui il feto è rimasto nell'utero senza vita.

La fase preliminare di questo studio prevede l'analisi di tessuti fetali umani provenienti da interruzioni legali di gravidanza, che verranno analizzati con tecniche microscopiche.

Le chiediamo quindi, qualora acconsenta, di esprimere un libero consenso all'utilizzazione a scopo scientifico del materiale fetale a seguito delle procedure di interruzione legale di gravidanza.

Il campione donato verrà contrassegnato con un numero di protocollo al fine di garantirne l'anonimato, e non sarà più in alcun modo ricollegabile al donatore.

Di tutto il materiale fetale prelevato, solo pochi frammenti delle dimensioni di alcuni millimetri verranno utilizzati per le successive analisi.

Il materiale non utilizzato verrà immediatamente distrutto, mentre il materiale utilizzato verrà distrutto al termine delle analisi.

Medico _____ Paziente _____

5.

CONCLUSIONS

RETROSPECTIVE PHASE

The main need emerged regards the possibility to "correct" the results of a first fetal thanatological evaluation based on macro- and microscopic characteristics according to fetal and maternal clinical conditions, in particular week of gestation and diseases. This consideration becomes even more important in consideration to the fact that the majority of intrauterine fetal death is associated to pathological conditions and/or prematurity. It will now be shown a summary of the main considerations for each area of study.

With regard to the **macroscopic analysis of the fetus**, the main critical points of the chronological evaluation can be so identified:

- skin color is affected not only by macerative phenomena, but also by gestational age (prematurity is associated with a deep red color) and by the presence of pathological conditions;
- the degree of skin maceration, the main criteria of fetal maceration, is influenced to an appreciable extent by the presence of diseases, both by accelerating it (hydrops, ascites, polyhydramnios, chorioamnionitis) and by retarding it (oligohydramnios, anhydramnios); in addition, it is important not to confuse a "false" maceration of traumatic origin with a real one;

- assessment of the cranial bones is affected to a considerable extent by the state of the brain: an early colliquation may distort the assessment in advance, and a condition of cerebral edema can act the opposite: a correct interpretation of the cranial bones mobility can be conducted only after demonstrating the anatomical integrity of brain structures;
- the evaluation of the serosa color and the presence of blood-serum flows in internal cavities had proved to be the less reliable criteria, greatly influenced by diseases and associated conditions.

Coming now on **histological analysis of fetal organs**, the following points of importance can be highlighted:

- the best correlation between death-birth time and loss of nuclear basophilia was recorded by evaluating the liver, the kidney cortical distal tubules, the adrenal gland mature cortex, the gastro-intestinal tract and the pancreas;
- nuclear pyknosis could be a useful predictor of death-birth time in the analysis of tracheal chondrocytes and renal glomeruli, where it was extensively observed not earlier than 24 hours after death;
- the poor fixation of the organ due to anatomical peculiarities or to the presence of bleeding or severe congestive states can cause artifacts in the stainability of the organ, not to be confused with a decreased maceration;
- it is conceivable that the presence of meconium in the intestinal tract may result in a stainability decrease of the inner half of the mucosa in

contact with the meconium for direct chemical action; even in this case it would however be an artifact unrelated to macerative phenomena.

Finally, regarding **placental histological analysis**, the following considerations can be done:

- the evaluation of physiological or para-physiological variations in placental anatomy (syncytial knots, mineralization of the basal membrane of the main villi) must be completely abandoned for the thanatological evaluation;
- the placental characteristics that best correlate with the death-birth time were leukocyte karyorrhexis and myocytolysis of the umbilical vessels wall;
- the natural degenerative evolution of placental structures and the different response to pathological insults compared to the fetus make difficult a possible modulation of the chronological evaluations based on the presence of other diseases or associated conditions.

These qualitative considerations can not be in this phase of the study further characterized by a quantitative point of view: for this type of evaluation is necessary to increase the number of the study population in order to subsequently proceed with an overall statistical analysis of data.

As far emerged is then strengthened by a general lack of temporal correlation by applying to the study cases the thanatological criteria existing in literature: at best 20% of the cases fail to be properly placed temporally. Similarly, for the thanatological definition of the most critical period often involved in medical-forensic litigation

(represented by the first hours after the death) there is no reliable criteria to distinguish intermediate timeframes below 12-24 hours.

PROSPECTIVE PHASE

The main novelty emerged from the TEM study was a vacuolization of cell structures with progressive diffusion with increasing time after death.

The main features of this phase are as follows:

- progressive qualitative increase of vacuolization from T2 to T9 , both in skin and muscle;
- skeletal muscle is a more resistant structure compared to the skin, as it shows a vacuolization with smaller extension;
- vacuolization can not be related to defects in construction or preparation of the samples, as cell membranes and other structures are always intact;
- mitochondria show inconstant and not time-dependent alterations;
- cell junctions are always perfectly preserved.

Ultimately, the distinguishing feature of this phase of the study, absolutely new in the literature, was the observation of a progressive cellular vacuolization with increasing time after death. This vacuolization is evident from the first minute after death, and in some cases becomes quite destructive after 20 hours.

Moreover, the progression of vacuolization has been analyzed from a quantitative point of view: semi-fine sections of skin samples were observed by optical microscope and were performed the count of the number of vacuoles in different fields. It was observed in this way a significant increase in the average number of

vacuoles from T1 to T8: in particular, at T1 (pick-up time) were counted an average of 46 vacuoles on three fields, while the medium number of vacuoles to T8 (4 hours) was 123 (total increase of 167.4%). An intermediate value has been counted at T3 (30 minutes), where the average number of vacuoles was 77 (+ 67.4% compared to T1).

Clearly, these results have been observed from the analysis of four cases only, and the population must be implemented.

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